

# Mycotoxins in Fruits and Vegetables



Edited by:  
Rivka Barkai-Golan  
Nachman Paster



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and Nachman Paster**



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525 B Street, Suite 1900, San Diego, CA 92101-4495, USA  
30 Corporate Drive, Suite 400, Burlington, MA 01803, USA  
84 Theobald's Road, London WC1X 8RR, UK

First edition 2008

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#### Library of Congress Cataloging-in-Publication Data

A catalog record for this book is available from the Library of Congress

#### British Library Cataloguing in Publication Data

A catalogue record for this book is available from the British Library

ISBN: 978-0-12-374126-4

For information on all Academic Press publications  
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Printed and bound in the USA

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# Preface

Fungi play a substantial role in spoilage of fruits and vegetables, because of their pathogenicity to the harvested products. During the various stages of pathogenesis, however, some of these fungi may generate different mycotoxins – secondary metabolites that are toxic to humans and animals that consume the products. Numerous studies, reviews and books have been dedicated to the wide area of mycotoxins in agricultural crops, and especially to those in dry agricultural products, which play a major role in international commerce. During the last decades, various fruits and vegetables that form part of our daily diet have been added to the list of products exposed to mycotoxin contamination.

The present book deals with the major mycotoxigenic fungi that attack harvested fruits and vegetables – *Aspergillus*, *Penicillium* and *Alternaria* species – and the mycotoxins produced by them in the host tissues, e.g., aflatoxins, ochratoxin A, patulin and alternaria toxins. Some of these mycotoxins are known to be carcinogenic, and most of them are highly stable during processing and, therefore, can reach the consumer. Thus, although consumers will reject a visibly rotten fruit, processed fruit products may still form a significant source of these mycotoxins, and pose a serious threat to human and animal health throughout the world.

The production of aflatoxins, primarily by *Aspergillus flavus* and *A. parasiticus*, has often been recorded in fruits grown under tropical and subtropical climates, where environmental conditions support growth of mycotoxigenic aspergilli and their subsequent aflatoxin production. Among the fruits typically contaminated by aflatoxins, we find dried figs, dates and citrus fruits.



An association between ochratoxin A and infected grapes was first reported in 1995, when ochratoxin was found in human serum, and wine was suggested to be the source. Ochratoxin A has received particular attention because of its association with cancer-promoting activity. Worldwide studies on ochratoxigenic fungi, primarily *A. carbonarius*, *A. niger* aggregate and other *Aspergillus* species, as well as on ochratoxin A contamination in grapes, dried vine fruits and grape-derived products, indicated that these products, and especially wines, can be important dietary sources of ochratoxin A for people who consume large amounts.

Patulin is a mycotoxin characteristic of infected fruits. Although *Penicillium expansum* is the main pathogen responsible for patulin production in apples, pears and various stone fruits, other *Penicillium* species that are involved in fruit and vegetable decay may produce a variety of mycotoxins during their life cycle in the host. These include ochratoxin A, citrinin, penicillic acid, citreoviridin, penitrem and other secondary metabolites such as chaetoglobosins, communesins, roquefortine C, expansolides, janthitrems and others, which may elicit toxicological effects in humans and animals.

*Alternaria* mycotoxins, including alternariol, alternariol methyl ether, altenen, tenuazonic acid and altertoxins, may be produced naturally in a wide range of fruits and vegetables, under both cold-storage and shelf-life conditions. Although *A. alternata* has been considered the major mycotoxin-producing species, other members of the genus, such as *A. citri*, *A. solani*, *A. longipes* and the *A. tenuissima*, *A. arborescens* and *A. infectoria* species groups, are also capable of producing these toxic contaminants in their hosts.

This book brings together for the first time subjects relevant to mycotoxin production and accumulation in fruits and vegetables, and ways for their prevention and control. Although each chapter constitutes a separate unit, the continuity of the chapters is relevant to a better insight of the diverse aspects of the whole subject.

The first two chapters deal with risk assessment and safety evaluation of mycotoxins, and with the economic aspects of mycotoxins in fruits and vegetables. These chapters are followed by descriptions of limits and regulations for mycotoxins in fruits and vegetables that have been established in many countries to protect the consumer from the toxic effects of mycotoxins.

Chapters 4 and 5 focus on the variety of factors affecting mycotoxin production in fruits, and describe the ability of mycotoxins to diffuse to some extent from the point of contamination into the adjacent healthy fruit tissues. Chapters 6, 7, 8 and 9 focus on the major mycotoxins produced by *Aspergillus*, *Penicillium* and *Alternaria* species in fruits and vegetables, with special attention to the molecular diversity of *Aspergillus* and *Penicillium* species.

Methods for detection of *Aspergillus*, *Penicillium* and *Alternaria* species are described in Chapter 10, and methods for detection and determination of ochratoxin A in grapes and wines and of patulin and *Alternaria* mycotoxins in their relevant host fruits and vegetables are described in Chapters 11, 12 and 13, respectively.

Chapters 14, 15 and 16 deal with chemical, physical and biological methods for suppressing the growth of mycotoxigenic fungi – methods which will indirectly lead to the prevention or suppression of toxin production – whereas Chapters 17 and 18 are dedicated to the direct control means for suppression and decontamination of mycotoxins in fruits and vegetables, and in their products.

Our special appreciation goes to each of the distinguished authors for contributing their up-to-date chapters that constitute both the foundation stones and the structure of the present book.

Rivka Barkai-Golan  
Nachman Paster

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# Risk Assessment and Safety Evaluation of Mycotoxins in Fruits

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## ABSTRACT

Relevant mycotoxins are signalled in fruits, with major problems in dried fruits as compared to fresh fruits. In order to study the risk of contamination by mycotoxins at global level, *Aspergillus* rot and ochratoxin A in grapes, *Aspergillus* and aflatoxins in pistachio nuts and peanuts, and blue mould rot and patulin in apples and pears were considered as case studies. They were chosen as representative of all fruits because the mycotoxins involved in the three host-pathogen systems depend on the field period, on both the field and the post-harvest period, and mainly on the post-harvest period, respectively. In the three case studies, the causal agent and symptoms, the occurrence of fungi and mycotoxins, and the ecology of fungi were described with all the details available. Then, data on crop distribution all over the world and meteorological conditions in the growing areas were collected and elaborated with a geostatistical approach to predict the level of risk for each mycotoxin in the considered crop; the outputs were drawn as risk maps that do not consider the effect of cropping systems.

The approach followed cannot be very precise, because of the global dimension and the simulated approach, but surely it represents a good base for safety evaluation for fruits produced all over the world. This can be used as a prototype approach and can be transferred to other crops and mycotoxins/diseases. Anyway, maps show high-risk areas somewhere in the world for all mycotoxins considered, mainly aflatoxins, that suggest high attention especially when the country of origin is a third country where fruits are probably not managed following the best practices.

## 1.1. MYCOTOXINS IN FRUITS

### 1.1.1. SIGNALLED MYCOTOXINS IN FRUITS

Mycotoxins may occur in raw agricultural products pre- and post-harvest. Cereals are among the most studied crops for toxin contamination, but fruits and their processed products may also represent a potential source of risk, with species belonging to *Aspergillus* and *Penicillium* worldwide recognised as the main concern.

Ochratoxin A (OTA), produced by species belonging to *Aspergillus* Section *Nigri*, was recently signalled in grape-derived products (Zimmerli and Dick, 1996), with origin in the vineyard (Battilani et al., 2003b); OTA is also a relevant problem in cocoa and coffee, where it also originates in the field but with potential increase post-harvest before fermentation or technological treatments (Magan and Aldred, 2005).

Aflatoxins (AFs), one of the most dangerous groups of mycotoxins, are mainly produced by *Aspergillus flavus* and *A. parasiticus*, belonging to *A. Section Flavi*, on several fruits, like peanuts, pistachio nuts, Brazil nuts and figs, signalled at high risk. On the other hand, almonds, pecans, walnuts and raisins are considered at lower AF risk (Jelinek et al., 1989). All these crops are mostly contaminated in the field, but conditions favourable for fungal growth during fruit storage can lead to an increase in mycotoxin level.

Patulin (PAT), a metabolite principally produced by *Penicillium expansum*, is probably the best-known toxin associated with fresh fruits like apples, pears, apricots, peaches and grapes; it is mainly considered a post-harvest problem.

Other mycotoxins may occur in fruits and vegetables although they do not represent a true toxicological risk. Worthwhile mentioning are *Alternaria*-toxins, produced by various *Alternaria* species, which are widely distributed in soil and on aerial plant parts and can be pathogenic to various fruits and vegetables. At least 20 species of *Alternaria* are known to synthesise toxic metabolites, but their natural occurrence is not considered relevant (Bottalico and Logrieco, 1998).

The concern about the cited mycotoxins is confirmed by EC regulation No. 1831/2003, where all contaminants in food were included and limits were fixed for OTA presence in unprocessed and processed cereals and coffee, dried vine fruits, grape juice and wine, for AFs presence in groundnuts, nuts, dried fruits, cereals, maize, several spices and milk, and for PAT in fruit juices, apple juices, spirit and fermented drinks derived from apple juice and solid apple products.

To summarise, almost all the mycotoxins of main concern in fruits originate in the field, during crop growth, and meteorological conditions are the key factors for risk assessment and safety evaluation. Some examples were taken into account as representative case studies and causal agents and symptoms, occurrence of fungi and toxins and ecology were described based on available data.

## 1.1.2. *ASPERGILLUS* ROT OF GRAPES

### 1.1.2.1. Causal agents and symptoms

The disease, particularly severe in the warmer grape-producing areas, as in southern Spain, France and Italy, in Greece, Egypt and Morocco, is caused by the so-called “black aspergilli”, fungi belonging to *Aspergillus* Section *Nigri*. Infected berries are generally pale at the first stage of the disease and then become black due to the conidial mass. The conidial heads may be seen with the naked eye and the spores are easily discarded when mature, resulting in soot-like deposits on adjacent berries (Snowdon, 1990). The fungi survives mainly on soil and the conidia are disseminated in vineyards by warm (25–30°C) air currents (Leong et al., 2006a). The infection happens via injuries, essentially in mature berries, and then under warm conditions it spreads throughout the bunch; colonisation and infection of bunches may also occur through injuries caused by careless handling. Mechanical cracks or wounds caused by insect or bird feeding may provide entry to berry tissue where fungi find an ideal habitat for their development; a correlation between *Lobesia botrana*, damaged berries and OTA contamination in the vineyard has been recently found (Cozzi et al., 2006).

*Aspergillus* Section *Nigri*, because of its black spores, are highly resistant to sunlight, and survive sun-drying, even if exposure for several days reduces spore viability (Leong et al., 2006b). Survival and subsequent growth of spores were prolonged at low temperatures and at available water ( $a_w$ ) below 0.6. Temperatures above 15°C at 0.6–0.9 $a_w$  were often more deleterious than at 1.0 $a_w$ . However, at 1°C and 1.0 $a_w$ , spores lost viability more rapidly than at lower  $a_w$ . As a consequence, increased incidence of black aspergilli in dry soils and from grapes in dry conditions may result partly from prolonged survival of spores (Leong et al., 2006c).

### 1.1.2.2. Occurrence of black aspergilli

*Aspergillus* Section *Nigri* includes several species difficult to be identified with traditional methods because differences are mainly based on the uniseriate and biseriate condition of the sterigmata of conidial heads, and on the size and roughness of the conidia. A simplified approach to manage surveys on these fungi suggest to consider only uniseriate (uniseriate sterigmata, never confirmed positive for OTA production), *A. niger* aggregate (biseriate sterigmata and with a low percentage of positive strains for OTA production) and *A. carbonarius*, easier to be identified due to the biseriate sterigmata and the very big conidia, with a high percentage of OTA producing fungi (Battilani et al., 2003b). Many efforts were devoted to develop molecular methods as a support for black aspergilli identification and a reliable real-time PCR assay was developed for *A. carbonarius* detection in grapes (Mulè et al., 2006); this is relevant in practice because *A. carbonarius* is considered as the main species responsible for OTA presence in grapes, dried vine fruits and wine, especially red wine.

Field surveys carried out in Italy showed that *A. niger* aggregate represent about 60 percent of the isolates, followed by uniseriates (21 percent) and *A. carbonarius* (19 percent). The most toxigenic strains proved to be *A. carbonarius*: about 60 percent of the isolates were OTA producers. *A. carbonarius* was more frequent in the South, but both in the South and in the North the percentage of positive isolates was high and included strains able to produce *in vitro* more than 100  $\mu\text{g}$  of OTA  $\text{kg}^{-1}$  of the medium (Battilani et al., 2003a).

Investigations carried out by Sage et al. (2002), showed that 6/11 grape samples collected from southern France were contaminated by potentially ochratoxigenic strains and all the 14 strains of *A. carbonarius* tested were able to produce OTA (up to 87  $\mu\text{g}$   $\text{kg}^{-1}$ ). From dried vine fruits in Argentina, *A. niger* proved to be the most common species, but only 3 of 293 isolates screened were OTA producers. On the other hand, *A. carbonarius* was less common, but 96 percent of the 48 strains tested produced OTA (Romero et al., 2005).

Geostatistical analysis, applied to the incidence of *Aspergillus* Section *Nigri* and *A. carbonarius* in Southern Europe and Israel for the 3-year period 2001–2003, showed that the highest incidence of black aspergilli was normally observed at harvesting. At this grape growth stage, spatial variability of black aspergilli was significantly related to latitude and longitude, showing a positive West–East and North–South gradient. Predictive maps of the incidence of infected berries showed the same trend in the 3 years, with highest incidence in 2003, followed by 2001 and 2002. The highest incidence was always observed in Israel, Greece and Southern France, associated with the highest incidence of *A. carbonarius*. The spatial distribution was strictly related to the meteorological conditions of the studied area (Battilani et al., 2006a).

### 1.1.2.3. Natural occurrence of ochratoxin A in grapes and wine

Ochratoxin A is a problem that originates in the vineyard (Battilani et al., 2004a); OTA is formed only prior to alcoholic fermentation (Zimmerli and Dick, 1996) and it is more commonly detected in red wines (54 percent) than in rosè (40 percent) and white wines (25 percent), with concentrations higher in red wines (Otteneder and Majerus, 2000). In addition, the amount of OTA was dependent on the latitude of the production region: the lower the latitude, the more frequent the occurrence and the greater the concentration (Otteneder and Majerus, 2000; Battilani et al., 2004a).

In Italy, 56 samples of red (38), rosè (8), white (9) and dessert (1) wine were contaminated with high levels of OTA ranging from  $<10$  to 7600  $\text{ng L}^{-1}$  with red wines more contaminated than rosè and white wines (Visconti et al., 1999). Additional data reported by Castellari et al. (2000) indicated the presence of OTA in samples of red wine from Italy (7/7, up to 500  $\text{ng L}^{-1}$ ) and Spain (1/1, 74  $\text{ng L}^{-1}$ ), and in samples of white wine from Italy (1/1, 12  $\text{ng L}^{-1}$ ) and France (1/1, 11  $\text{ng L}^{-1}$ ). This tendency was also confirmed in 2001 (Pietri et al., 2001), when OTA was found in 14/96 red wines (up to 3200  $\text{ng L}^{-1}$ ) and in 6 out of 15 special dessert white wines (up to 3800  $\text{ng L}^{-1}$ ), with the distribution of OTA higher in wines produced in southern regions.

In Spain, OTA was found in 267 samples of wines of different types and 18 samples of grape products. Contamination was evident in 92, 91 and 65 percent of red, rosé and white wines with mean OTA concentration of 54, 31 and 20 ng L<sup>-1</sup> (Burdaspal and Legarda, 2000). In addition, OTA was detected in 74 percent of 47 samples of sherry wines (mean 40 ng L<sup>-1</sup>), in 83 percent of 12 samples of sparkling wines (mean 12 ng L<sup>-1</sup>) and in 94 percent of 16 samples of dessert wines (mean 1000 ng L<sup>-1</sup>). In a preliminary study by Filali et al. (2001), all of the 30 Moroccan samples of wine were contaminated with OTA (up to 3200 ng L<sup>-1</sup>). Immunoaffinity HPLC of 31 samples of red wine originating from Mediterranean countries and 15 samples of vinegar revealed that 72 percent of the wine samples and 100 percent of the vinegar samples were contaminated with OTA (up to 3.4 and 0.25 ng mL<sup>-1</sup>, respectively) (Markaki et al., 2001). In southern France, 8/11 musts from grape samples were contaminated with OTA (up to 460 ng L<sup>-1</sup>), and there was a strong correlation between the presence of *A. carbonarius* on grapes and OTA in musts (Sage et al., 2002). The average levels of OTA in locally produced red and white wines in Cyprus in 1999 were 0.12 and 0.2 µg L<sup>-1</sup> (Ioannou-Kakouri et al., 2001). Eltem et al. (2003) showed that 15 out of 52 analysed grape samples from various Turkey vineyards were positive for OTA, with a concentration up to 1.5 µg kg<sup>-1</sup>. In addition, the same authors reported an OTA contamination of 20 out of 61 raisin samples (from 0.1 to 25 µg kg<sup>-1</sup>).

A different susceptibility to *A. carbonarius* infection and OTA contamination of 12 different grape varieties has been shown by Battilani et al. (2004b). In particular, the incidence of colonised berries and their OTA contents were low in “Bianco d'Alessano”, “Pampanuto” and “Uva di Troia”. In contrast, “Cabernet Sauvignon”, “Trebiano” and “Verdeca” showed high fungal incidence and OTA content.

#### 1.1.2.4. Ecology of black aspergilli

Spore germination is rapid and achieved in less than 24 hours at temperatures above 25°C, over a wide  $a_w$  range of 0.90–0.99; germ tube extension of the germinated spores reflect this. A more rapid germination and establishment on grape tissue than grape skin even at 85 percent RH suggests that damage or any wounds may provide a very rapid entry point for spores present on the surface of the grape berries. This also indicates that conducive conditions for germination cover a wide temperature and  $a_w$  range and this could occur easily overnight in the canopy of a vineyard mainly on the surface of ripening grapes (Mitchell, 2006).

Temperatures in the range of 10–37°C were tested and optimum temperatures for growth of *Aspergillus* Section *Nigri* were between 30 and 37°C. Water activity levels ranging from 0.90 to 0.995 are suitable and optimum  $a_w$  for growth was 0.98 (Belli et al., 2004a).

Most *A. carbonarius* strains grew optimally at 30–35°C, with no growth at <15°C. The optimum  $a_w$  for growth varied from 0.93 to 0.99, depending on the strain, with the widest  $a_w$  tolerance at 25–30°C. There was no direct relationship among growth, environmental factors and country of origin of



individual strains even if the amount of OTA produced varied markedly between isolates from different countries. For example, isolates from Spain produced  $4\text{--}5\text{ }\mu\text{g g}^{-1}$ , while strains from Italy and Portugal produced  $<1\text{ }\mu\text{g g}^{-1}$  medium at optimum  $a_w$  conditions.

Optimum conditions for OTA production varied with strain, but the range of optimal OTA production was at  $15\text{--}20^\circ\text{C}$  and  $0.95\text{--}0.98a_w$  for *A. carbonarius* (Mitchell et al., 2004; Belli et al., 2005; Esteban et al., 2006; Leong et al., 2006d) while *A. niger* aggregate strains achieved maximum OTA levels mainly at  $20\text{--}25^\circ\text{C}$  (Esteban et al., 2004). Ochratoxin production by *A. carbonarius* was detected mainly at  $0.97a_w$  (Leong et al., 2006d; Valero et al., 2006a). Earlier production was detected at  $30^\circ\text{C}$  (optimum for growth), whereas maximum OTA concentration was found at  $20^\circ\text{C}$ . Ochratoxin production was detected from mycelium that was only a few days old and highest level was found when mycelium was 4–7 days old at  $0.97a_w$ . OTA diffusion was observed at  $0.92a_w$  and  $20^\circ\text{C}$  (Valero et al., 2006a).

*Aspergillus carbonarius* is able to grow and produce OTA over a wide pH range at high or low temperature (Esteban et al., 2005). The effect of different pH levels on growth is important as the grape flesh and grape juice are all very acidic ( $\text{pH} < 4.5$ ). Growth of *A. carbonarius* was better at pH 4 and 7 than at pH 2.6, regardless of the  $a_w$  level. This shows that very good establishment can occur in the region of pH 4, commonly achieved in grape-based products. Although we found that higher concentrations of OTA were produced at pH 7 than 4.5, the neutral pH is not a condition which normally exists in grape juice, wine or grape products. The pH of vine fruits is about 2.7–4.5; the higher production of OTA at pH 2.8 and 7 may reflect marginal environmental conditions for growth and this stress could explain the higher production at these pH levels than at 4.5 (Mitchell, 2006).

Competing mycoflora may act as a control factor against OTA accumulation at  $30^\circ\text{C}$ , but not at  $20^\circ\text{C}$  which correspond to optimal temperature for OTA production (Valero et al., 2006b).

The relevance of ecological conditions on OTA production and their possible use in OTA prediction was confirmed by field data. Using a discriminant equation, with summation of degree-day and of rain in the period between veraison and ripening as input, vineyards were correctly classified for OTA contamination and this approach is also promising for prediction purposes (Battilani et al., 2006b).

### 1.1.3. *ASPERGILLUS FLAVUS* AND *A. PARASITICUS* AND AFLATOXINS IN NUTS

#### 1.1.3.1. Causal agents and symptoms

*Aspergillus* is among the most ubiquitous fungal genera, mostly including saprophytic species that colonise plant debris or deteriorated agricultural commodities, but also a few strains that are able to colonise living plants.

Besides their economic significance as spoilage agents of plant products, particularly grains and legumes, some species are capable of producing mycotoxins which are able to elicit tremendous effects on human and animal health. Aflatoxins are synthesised by some strains of *A. flavus*, which produce essentially AFB<sub>1</sub> and AFB<sub>2</sub>, and by most strains of *A. parasiticus*, which also form AFG<sub>1</sub> and AFG<sub>2</sub>. Of these, four main AFs, AFB<sub>1</sub> and AFG<sub>1</sub> occur most frequently and in the largest amounts in plant products.

Symptoms may first appear on or in peanut pods in the soil before digging, especially when plants are stressed by drought. *Aspergillus* colonies develop on pods and seeds that are overmature or on which damage provides openings for the fungus. Following digging, during the curing process, additional pods and seeds may become invaded, especially the immature pods (Porter et al., 1984).

Preharvest damages, like early hull splitting and insect damages, were found correlated to *Aspergillus* infections and AFs contamination in pistachio nuts. Early splitting causes shell discoloration; this symptom, together with small size, might be an indicator of high-risk nuts for AFs contamination (Schatzki and Pan, 1997).

#### 1.1.3.2. Occurrence of *A. flavus* and *A. parasiticus*

Aflatoxin-producing strains of *A. flavus* and *A. parasiticus* are distributed worldwide in soil and air and are classifiable as both storage fungi and field fungi, since some strains are phytopathogenic. These can infect plants in the field and then colonise harvested or stored plant products.

Pistachio nuts were sampled periodically from Californian orchards prior to harvest to determine when *A. flavus* infected the developing nuts and if AFs were present. *A. flavus* was not isolated from nuts in July and from only 0.3 percent of the nuts in August. However, 11.8 percent of nuts were infected at harvest (15 September). The increased infection was correlated with the exposure of the nut as the endocarp (shell) and often the mesocarp (hull) were split during maturation. At harvest, 28 percent of the insect-damaged nuts were infected compared with only 6 percent of the undamaged nuts. When tested for AFs, 70 percent of the *A. flavus* isolates produced AFB<sub>1</sub>. However, AFs were not detected in 29 nut samples (Thomson and Mehdy, 1978).

Aflatoxin-positive samples of pistachios from Iran were analysed. The incidence of AF-producing strains of *A. flavus* was higher in these samples than these in AF-negative samples of pistachios (Ichinoe et al., 2001).

Average air and soil temperatures of 28–34°C were favourable for AFs contamination in peanuts. In general, early sowing produced greater pod yields, as well as less infection and lower AF concentration. There were negative linear relations between infection ( $r^2 = 0.62$ ) and the average simulated fraction of extractable soil water (FESW) between flowering and harvest, and between AF concentration ( $r^2 = 0.54$ ) and FESW in the last 25 days of pod-filling. This field study confirms that infection and AFs concentration in peanuts can be related to the occurrence of soil moisture stress during pod-filling when soil temperatures are near optimal for *A. flavus* (Craufurd et al., 2006).

### 1.1.3.3. Natural occurrence of aflatoxins in peanuts and pistachio nuts

Aflatoxins can accumulate in many important agricultural commodities and among plant products, nuts (e.g., peanuts, hazelnuts, pistachio nuts and almonds) and dried fruits from tropical and sub-tropical areas are those with major risk of AF contamination.

Eighty-seven samples of groundnuts and groundnut products marketed in the region of Marilia, Sao Paulo, Brazil were analysed between 1999 and 2001 to identify and quantify AFB<sub>1</sub> and AFG<sub>1</sub>. Aflatoxins were found in 56 (64.4 percent) of the samples and 34 (39.1 percent) exceeded the limits of the Brazilian legislation for AFB<sub>1</sub> + AFG<sub>1</sub> of 30 µg kg<sup>-1</sup>. The AFs concentration in the samples varied from 3 to 1659 µg kg<sup>-1</sup> and the average of positive samples was 306 µg kg<sup>-1</sup>. In 31 (35.6 percent) samples, AFs were not detected. High levels of AFs were observed during high temperature and rainfall levels (Shundo et al., 2003).

### 1.1.3.4. Ecology of *A. flavus* and *A. parasiticus*

Infection of peanuts by *Aspergillus* occurs under both pre- and post-harvest conditions. Pre-harvest infection and consequent AF concentration is more important in the semi-arid tropics, especially when drought occurs in the last 20–40 days of the season; 30–50 days of drought period and a mean podding zone temperature of 29–31°C were shown as the conditions for pre-harvest contamination (Cole et al., 1985 and 1989; Doner et al., 1989); drought without high soil temperature does not result in contamination. Shorter periods of drought (<20 days) and drought early or late in the season, as well as cooler or warmer temperatures than 29–31°C, even if drought is imposed, also result in lower concentration of AF. These conditions have not been verified in West Africa, where AF is a major problem (Craufurd et al., 2006).

## 1.1.4. BLUE MOULD ROT OF APPLE AND PEAR

### 1.1.4.1. Causal agents and symptoms

The disease is caused by *Penicillium expansum*, a pathogen which is generally considered to be a wound parasite. Mould growth normally occurs only where the surface tissue of fruits has been damaged. Infection commonly follows insect or storm damages during pre-harvest, rough gathering at harvest or strong washing and sorting procedures post-harvest. Lenticels susceptibility, as penetration site, is chiefly determined by maturity, with over-mature or long-stored fruits very conducive and fertiliser treatments and light intensity playing a role (Snowdon, 1990).

The symptoms start with soft watery brown spots which enlarge rapidly at 20–25°C; blue-green spores are formed on the lesion surface in humid conditions. Soft rotten flesh and healthy tissues are clearly separated (Snowdon, 1990).

During storage, infection can occur even at 0°C, but decay proceeds slowly during cold storage, and usually develops rapidly only when fruits are returned to warm temperatures. The species includes strains with different pathogenicity, as determined by lesion diameter on fruits, and with different capacities to produce PAT, from 2 to 100 mg kg<sup>-1</sup> (Paster et al., 1995).

#### 1.1.4.2. Occurrence of *P. expansum*

No studies are available on the natural occurrence of *P. expansum* in apples at harvesting, probably because this is considered a post-harvest disease. Only a survey carried out in Canada on rotten apples showed 68 percent of fruits with the fungus, but only 46 percent with PAT (Harwig et al., 1973).

#### 1.1.4.3. Natural occurrence of patulin in apples

Under laboratory conditions, PAT can be produced by a variety of different moulds on apples, grape juice and grains, but in natural conditions PAT is essentially known as a metabolite of *P. expansum* contaminating apples and apple juice. Patulin is stable in apple juice, while when fruit juice is converted to cider it does not contain PAT because it is destroyed by alcoholic fermentation. High levels of PAT (up to 250 mg kg<sup>-1</sup>) have been reported in 52 percent of 104 Spanish apple samples (Jelinek et al., 1989), but the level of contamination in the juice is usually lower, probably due to the disposal of apples affected by rot. To this regard, the occurrence of PAT was reported in 43 out of 100 samples of Spanish apple juices at concentrations of 10–170 ng mL<sup>-1</sup> (Prieta, 1994). Investigation carried out on Italian products showed PAT in all five samples of apple juice examined (up to 60 ng mL<sup>-1</sup>), in five out of six pear juices (up to 25 ng mL<sup>-1</sup>), in two out of six peach juices (up to 3 ng mL<sup>-1</sup>), in one out of three apricot juices (12 ng mL<sup>-1</sup>) and in more than 50 percent of 20 samples of jam (up to 75 ng g<sup>-1</sup>) (Valletrisco et al., 1983). A very high incidence (100 percent) of PAT, even though at low levels (5–75 ng mL<sup>-1</sup>), was found in 44 samples of Turkish apple juice (Karadenizm and Eksi, 1997), but a similar survey carried by Gökmen and Acar (1998) showed higher PAT contamination of 215 apple juices from Turkey with 100 percent incidence and toxin range of 7–376 ng mL<sup>-1</sup>. In France, PAT was found in 100 percent (up to 610 ng g<sup>-1</sup>) and 69 percent (up to 300 ng g<sup>-1</sup>) of 27 samples of concentrated apple juices, and 13 samples of apple cider (Jelinek et al., 1989). Laidou et al. (2001) reported the formation of PAT in pear inoculated with *P. expansum* and its diffusion in the apparently sound flesh, in concentration surpassing the accepted maximum European limit (50 µg kg<sup>-1</sup>). Investigation carried out by Martins et al. (2002) on the presence of mycotoxins in 351 samples of seven different varieties of apples with small rotten areas, collected throughout Portugal, revealed the occurrence of PAT (up to 80.5 mg kg<sup>-1</sup>) in 89 percent of the examined samples. It can be detected in apples with small rotten areas available on the market (groceries and supermarkets); differences among cultivars were noticed (Martins et al., 2002). Patulin contamination was detected at significantly higher levels in organic than

in conventional apple juices in surveys carried out in Belgium and Italy (Baert et al., 2006; Piemontese et al., 2005).

The removal of contaminated apples from the initial processing line during apple juice production has to be considered seriously. Mean PAT concentration in non-processed apples can be relevant and it can be significantly reduced by an initial water wash step (till –80 percent) followed by further reduction after the removal of rotten and damaged apples (further –10 percent). During the pre-concentration stage, a relevant increase of PAT content was observed, probably due to total solids concentration (Brix). The combined depectinisation/charcoal/ultra-filtration stage yielded a significant decrease in mean PAT concentration probably due to the adsorption on the activated charcoal. No further removal of PAT occurred during the remainder of the juicing process (Leggott et al., 2000).

#### 1.1.4.4. Ecology of *Penicillium expansum*

Growth of *P. expansum* was prevented at low  $a_w$  values (0.83–0.85) and outside the range of 10–23°C, but in unpreserved apple or apple products ( $a_w > 0.97$ ) left at room temperature, growth may occur in a time as short as 1-day, probably leading to mycotoxin accumulation in a few days (Marin et al., 2006).

The growth curve of *P. expansum* on apple puree agar medium was characterised by a short lag phase followed by a linear growth. A decrease in sucrose, glucose and fructose content in the medium was observed, with the reduction in sucrose being more pronounced. In the first phase of growth, *P. expansum* exhibited exponential production of PAT and it increased with the increase of pH, while no correlation between the sugar content of apples and PAT production was recorded (Baert et al., 2004).

*Penicillium expansum* isolates originating from across Ontario, Canada, produced widely differing levels of PAT, ranging from 0 to  $>6 \text{ mg g}^{-1}$  by dry mycelial weight. Extensive fungal growth and higher PAT levels (538–1822  $\mu\text{g mL}^{-1}$  on day 14) in apple ciders were associated with incubation at 25°C, although potentially toxic PAT levels (75 to 396  $\mu\text{g mL}^{-1}$  on day 24) were also found in refrigerated ciders (4°C) inoculated with *P. expansum* (McCallum et al., 2002).

Patulin production was totally inhibited when the fungi were grown in apples stored under 3 percent  $\text{CO}_2$ /2 percent  $\text{O}_2$  (25°C) (Paster et al., 1995) and  $\text{SO}_2$  had the greatest inhibitory effect on mycelium growth and PAT production *in vitro* (Podgorska, 1992).

## 1.2. GLOBAL CROPS DISTRIBUTION AND METEOROLOGICAL CONDITIONS

Referring to the examples considered, the growing period is crucial for OTA production in grapes, both pre- and post-harvest are relevant for AFs accumulation in peanuts and pistachio nuts, while post-harvest is considered

as fundamental for PAT in apples/pears and field data are practically absent. A geostatistical approach was followed to describe the risk of contamination all over the world based on meteorological data for crops and mycotoxins that are totally or partially produced in the field. This is to simulate the effect of those variables, meteorological variables, that we cannot manage. No simulation approach was considered for the post-harvest period where ecological parameters can be managed. In fact, in several countries protocols for post-harvest management were implemented and accumulation of toxin is no more a relevant problem; the improvement of post-harvest conditions is still a problem in the third countries, not easy to be solved but no help can be found in a simulation approach.

### 1.2.1. CROP DISTRIBUTION

Data on world distribution of selected crops were extracted from FAOSTAT, a database managed by FAO and available at <http://faostat.fao.org/site/291/default.aspx>. FAOSTAT provides access to over 3 million time-series and cross-sectional data relating to food and agriculture; it contains a full matrix of integrated and compatible statistics coverage of 200 countries, 15 years, and more than 200 primary products and input items related to production, trade, resources, consumption and prices. The list of countries where a certain crop is grown was obtained. It was associated with a map where the administrative boundaries of countries were drawn and using a digital model of altitude and latitude, the unuseful parts of each country for the selected crop were excluded.

The Shuttle Radar Topography Mission (SRTM) obtained elevation data on a near-global scale to generate the most complete high-resolution digital topographic database of earth. SRTM consisted of a specially modified radar system that flew onboard the Space Shuttle Endeavour during an 11-day mission in February 2000. SRTM is an international project spearheaded by the National Geospatial-Intelligence Agency (NGA), NASA, the Italian Space Agency (ASI) and the German Aerospace Center (DLR).

#### 1.2.1.1. Global grapevine distribution

Grapevine has been grown since ancient times (Neolithic) in Europe, Middle East, Caucasus and North Africa and later it was spread over all continents, in particular North (California) and South America (Argentina, Chile, Brazil), Australia, South Africa and China. This plant is able to adapt to very different environments, even if some limitations exists. The cropping area is included between 30° and 50° latitude, both north and south, and on altitude up to 1000 m above sea level, with temperatures above -15°C (Fregoni, 2005).

### 1.2.1.2. Global peanuts distribution

Major countries for peanuts growing, with more than a million hectares, are India, China and Nigeria, followed by countries with half a million hectares (Senegal, Sudan, Zaire, Indonesia and USA) and with less than half a million hectares (Myanmar, Chad, Vietnam, Mozambique, Burkina Faso, Mali, Uganda, Argentina, Zimbabwe, Cote D'ivoire, South Africa, Pakistan and Thailand). Although India has the largest area of groundnuts, China produces more than any other country in the world. Based on available data, the cropping area of peanuts was considered to be included between 45° latitude north and south and on altitude up to 600 m above sea level.

### 1.2.1.3. Global pistachio nuts distribution

Pistachios are truly Mediterranean by adaptation; they thrive in the hot, dry, desert-like conditions. Trees are highly disease prone, and rain or even high humidity during the spring and summer promotes severely debilitating diseases. Chilling requirement is relatively long, 1000 hour. Thus, arid climates with rather cool, prolonged winters are needed for pistachios. Pistachios thrive in areas which have winters cool enough to break bud dormancy and hot, long summers. They are drought resistant and very tolerant to high summer temperatures, but cannot tolerate excessive dampness and high humidity. The tree has about the same cold resistance as almonds and olives but flowers later in spring than almonds. Chill requirements are estimated at 600–1500 hours.

Pistachio trees are fairly hardy in the right conditions, and can survive temperature ranges between  $-10^{\circ}\text{C}$  in winter to  $40^{\circ}\text{C}$  in summer. They need a sunny position and well-drained soil, they do poorly in conditions of high humidity and are susceptible to root rot in winter if they get too much water and the soil is not sufficiently drained. Long hot summers are required for proper ripening of the fruit. Based on available data, the cropping area of pistachio nuts was considered included between 40° latitude north and south and at altitude between 300 and 900 m above sea level.

## 1.2.2. METEOROLOGICAL DATA

### 1.2.2.1. Climatic database

The relevant role of meteorological/ecological parameters was confirmed in all the diseases described. Consequently, the risk of mycotoxin contamination in the selected fruits can be assessed, at world level, starting from the description of meteorological conditions in the crop growing areas.

Reliable data collected from meteorological stations well distributed on the territory and with sufficiently long historical series are necessary to describe the areas and predict the risk of mycotoxin occurrence with a robust base.

The selected database was FAOCLIM and it was downloaded from the Internet link: [ftp://ext-ftp.fao.org/SD/SDR/Agromet/New\\_LocClim/](ftp://ext-ftp.fao.org/SD/SDR/Agromet/New_LocClim/). It is maintained

TABLE 1.1 World meteorological data available in FAOCLIM.

Variable	No. of stations	EMD <sup>a</sup>
Mean temperature	20828	48.36
Mean minimum temperature	11550	64.94
Mean maximum temperature	11544	64.96
Rainfall	27375	41.71

<sup>a</sup>EMD is the effective maximum distance (in Km) to the nearest station; it is half the distance between two stations if the stations were homogeneously distributed.

TABLE 1.2 Inventory of time series (number) of variables considered according to continents.

	Africa	Asia	America	Europe	Oceania	Antarctic	World
Mean temperature	605	1256	2765	765	515	90	5996
Rain	3395	2172	5611	1389	915	48	13530

by the FAO Environment and Natural Resources Service (SDRN) in the Sustainable Development Department. FAOCLIM contains monthly data for up to 14 observed and computed agro-climatic parameters, but average temperature and rainfall are the most commonly available parameters (Table 1.1).

Thirty per cent of the series refer to rainfall and 30 percent to temperatures; the average length of the series is around 50 years. Some series exceed 200 years, while the longest rainfall series covers 299 years. On the other hand, 185 rainfall series include only 1 year. Eighty per cent of the series exceed 20 years and about 45 percent exceeded 50 years. Ninety per cent of rainfall time series and 86 percent of temperature series have no gaps, that is on average a missing data items occur over 10 years for rainfall time series. The time series differ significantly in relation to the continent (Table 1.2).

Temperature and rain data were extracted from FAOCLIM for all the stations placed in the area of cultivation of the selected crops and for the period considered relevant for mycotoxin production.

1.2.2.2. Data relevant for considered crops

Taking into account the limits of grapevine, peanuts and pistachio nuts growing areas, and based on previous studies suggesting the month preceding ripening as a crucial month for OTA (Battilani et al., 2006a and 2006b) and AFB<sub>1</sub> accumulation (Thomson and Mehdy, 1978), data on mean daily temperature and total rain, in August in the northern and in February in the southern hemisphere, were extracted from FAOCLIM.

1.2.2.3. Geostatistical data analysis

The above-mentioned data were elaborated using a geostatistical approach to predict data in unavailable places. The geostatistical approach is appropriate



when variables are continuous and strictly dependent on the geographic site and time. In particular, Kriging method was applied allowing to obtain the best linear unbiased estimators of spatially dependent data (Isaaks and Srivastava, 1989). Kriging is a means of local averaging of the weights of data from sampled locations surrounding an unsampled location based on statistical similarity to the unsampled locations; it gives unbiased estimates minimising the estimated variance. This approach gives the elements to differentiate variables on a geographical basis and, as user-friendly output, to draw maps in which the gradient of the variables is highlighted.

The parameters, temperature and rain in the selected period and areas were inputted into the geostatistical analysis module of ArcView 8.2 (2002) and ordinary Kriging was applied to predict points not sampled on the whole geographic area considered.

Cross validation was applied to check the goodness of estimates obtained by Kriging (Davis, 1987). Cross validation consist of applying the spatial model selected to predict a value in each sampling point and then compare predicted and observed data; the difference between the two values is the error of estimate. Then, mean and variance of errors are computed and compared to 0. Linear regression between predicted and observed data was also computed using SPSS (Statistical Package for Social Science, ver. 11.5.1, 2002); the determination coefficient ( $R^2$ ) represent the proportion of variance explained by the Kriging model and could be considered a good index to judge the efficiency of the model in representing data. The results of spatial analysis were represented with raster maps with the administrative boulder of world countries as cartographic base. A raster dataset is made up of cells having all the same size; each cell, or pixel, is a square that represents a specific portion of an area. Cells are arranged in rows and columns, an arrangement that produces a Cartesian matrix. All locations in the studied area are covered by the matrix and a specific value is assigned to each cell by Kriging interpolation process.

Maps of rain and temperature were drawn using the Kriging interpolation method and their reliability was confirmed by cross validation. The errors were acceptable for both parameters, with temperature better estimated than rain, which is known and due to the relevant variability of the latter parameter. Regression analysis of errors was highly significant ( $P \leq 0.01$ ) with  $R^2$  always higher than 0.70; the Kriging models found were efficient because they explained a relevant part of variance of considered variables.

## 1.3. GLOBAL RISK MAPS FOR MYCOTOXINS IN FRUITS

### 1.3.1. RISK OF OCHRATOXIN A CONTAMINATION IN GRAPES

Data on temperature and rain were ranked in order to define homogeneous areas. Based on the results of previous researches (Battilani et al., 2006b), the

TABLE 1.3 Risk of OTA accumulation in grapes associated to combination of mean temperature (°C) and summation of rain (mm) during August and February respectively, in northern and southern hemispheres.

Rain temperature	0–5	5–35	35–75
15–20	L	L	L
20–25	M	H	M
25–30	L	M	L

H – high; L – low; M – medium.

risk associated to combinations of temperature and rain was classified as follows (Table 1.3).

A map was drawn painting the growing areas with different colours based on this definition of risk levels (Fig. 1.1).

In the northern hemisphere, high-risk areas are placed in Europe, mainly around the Mediterranean basin, with highest risk associated to Hispanic peninsula, south Italy, Greece and Turkey.

Data collected in a specific European project (WINE-OCHRA RISK; QLK1-CT-2001-01761) substantially confirmed the figure drawn in the map (Belli et al., 2004b; Battilani et al., 2006b; Guzev et al., 2006; Tjamos et al., 2006), with some exceptions, related to a lower risk in Portugal (Serra et al., 2006) and a higher risk in southern France (Bejaoui et al., 2006).

Countries faced to the southern Mediterranean seem not to be included among the high-risk areas, probably because of very high temperature and very low rain. It is confirmed by data produced in Israel (Guzev et al., 2006) while more relevant values were reported by Filali et al. (2001) in Morocco.

In North America the areas characterised by high risk are placed in the south-west, in particular in California, which is characterised by climatic conditions very similar to some Mediterranean countries. Few data are available for the United States and they signalled no quantifiable presence of OTA, but the area of grape production was not defined (Ng et al., 2004).

The Austral hemisphere, South Africa and Australia have a high-risk area, while South America is less exposed. This is confirmed by Chulze et al. (2006), who found very low levels of OTA in Chilean, Argentinean and Brazilian wines. Low levels were also reported by Leong et al. (2006a) for Australia and by Shephard et al. (2003) for south Africa.

1.3.2. RISK OF AFLATOXIN B<sub>1</sub> CONTAMINATION IN PEANUTS

The approach followed for OTA in grapes to define homogeneous areas using data on temperature and rain, based on the results of previous researches, was applied also for peanuts and maps were consequently drawn (Table 1.4).

According to the simulation (Fig. 1.2), low-risk areas are mainly placed in northern hemisphere.

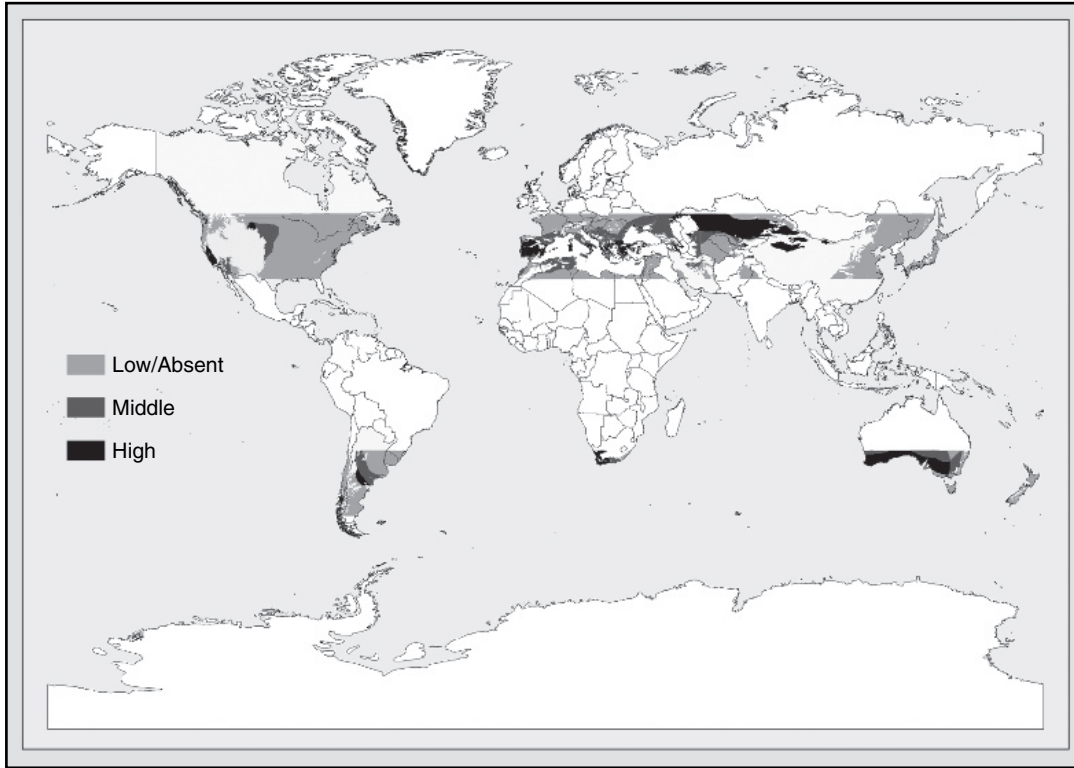


FIGURE 1.1 Prediction map of ochratoxin A risk in grapes growing areas on a global scale. The risk was computed with a geostatistical approach using the combination of mean temperature ( $^{\circ}\text{C}$ ) and summation of rain (mm) during August and February, respectively, in northern and southern hemispheres.

TABLE 1.4 Risk of AFB<sub>1</sub> accumulation in peanuts associated to combination of mean temperature (°C) and summation of rain (mm) during August and February, respectively, in northern and southern hemispheres.

Rain temperature	0–35	35–100	100—300	> 300
<20	L	L	L	L
20–25	L	M	M	L
25–30	M	H	H	M
>30	L	M	M	L

H – high; L – low; M – medium.

European peanuts growing areas are very limited, with around 500 Ha, but they are characterised by low-medium risk and this is in agreement with available field data. In Spain, a total of 33 commercial samples of unshelled peanuts, and 35 of shelled groundnuts collected in Madrid during May 1998 showed 21.2 and 57 percent of positive samples respectively, and all these positive samples were below the maximum legal limit according to both the current Spanish legislation and the EC regulations (Burdaspal and Legarda, 1998).

Countries in north and west Africa, in particular those facing the Mediterranean basin, were also described as low-medium risk areas and it was confirmed by Sangare-Tigori et al. (2006), who found low AFB<sub>1</sub> concentration in peanuts samples. On the contrary, the south-Saharan areas and the centre-south coasts, along Tanzania, Mozambique and Madagascar, are characterised by high risk of AFB<sub>1</sub> contamination.

Low risk can also be seen in maps of the Middle East, Arabic peninsula and central Asia, while high risk was associated with India and South Eastern Asia. Surveys on peanut-derived products produced in Indonesia confirmed the relevant presence of AFs. Indonesian groundnut-chilli sauce had the highest incidence of AFs contaminated samples (75 percent), followed by traditional snacks (45 percent), groundnut butter (40 percent), flour egg-coated groundnut (37 percent) and groundnut cake (30 percent). Fried and roasted groundnuts were found to contain AFs at relatively lower percentages of 9 and 8 percent, respectively (Razzazi-Fazeli et al., 2004).

A survey conducted in Tarai regions of Nainital, Uttaranchal, India showed 97 ppb of AFB<sub>1</sub> in groundnuts as average concentration, with 425 ppb as the highest level and 27.5 percent of the samples exceeding 20 ppb (Harish-Kumar et al., 2002). Samples of groundnut kernel (unroasted 102 and roasted 109) and groundnut cake (108), obtained from distributors and retailers of two districts of Uttar Pradesh during the period November 1995–April 1996, were screened for AFs. Groundnut samples obtained from distributors contained only low levels (24–323 ppb), while higher concentrations were detected from the retailers (108–742 ppb).

High contamination was mapped in Japan, in almost all Australia and in America. Surveys suggested a lower risk in Japan, AFs being detected in 10 of 21 peanut butter samples, with 2.59 µg kg<sup>-1</sup> as the highest concentration of AFB<sub>1</sub>

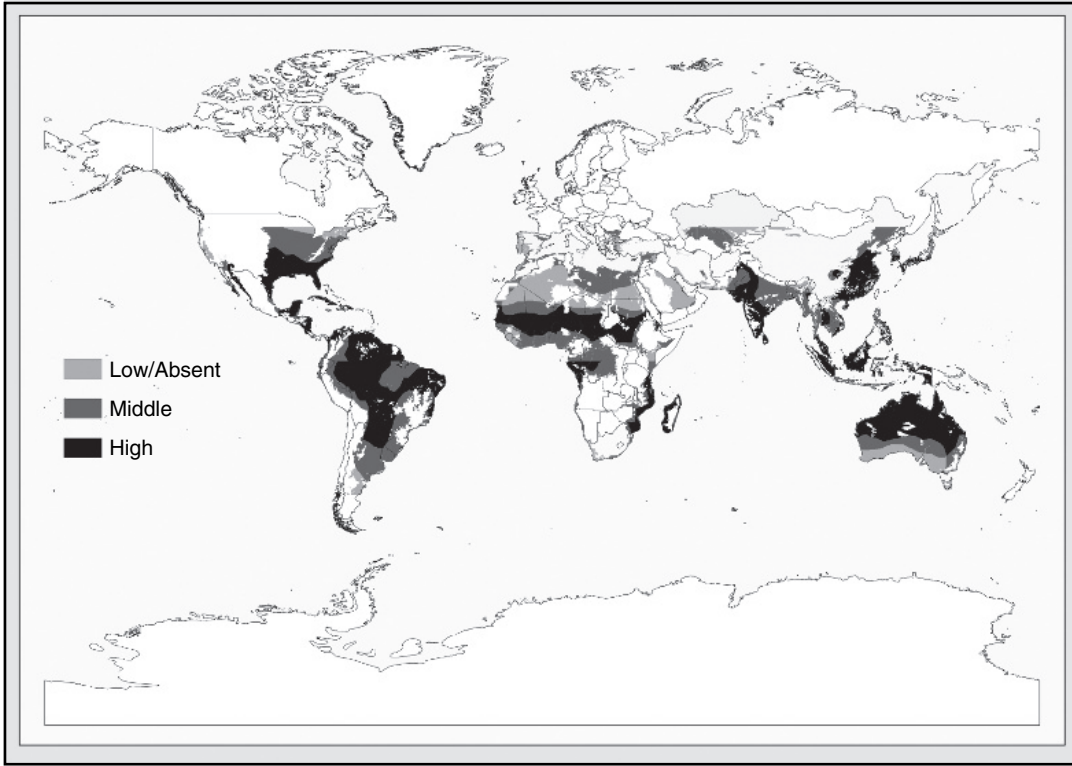


FIGURE 1.2 Prediction map of aflatoxin B<sub>1</sub> risk in peanuts growing areas on a global scale. The risk was computed with a geostatistical approach using the combination of mean temperature (°C) and summation of rain (mm) during August and February, respectively, in northern and southern hemispheres.

TABLE 1.5 Risk of accumulation of AFB<sub>1</sub> in pistachios associated with combination of mean temperature (°C) and summation of rain (mm) during August and February, respectively, in northern and southern hemispheres.

Rain temperature	0–35	35–100	100–300	> 300
<20	L	L	L	L
20—25	M	M	L	L
25—30	H	H	M	L
>30	M	M	L	L

H – high; L – low; M – medium.

(Sugita-Konishi et al., 2006), and confirmed the high risk in Brazil, where the levels below or equal to the Brazilian regulation (30 µg kg<sup>-1</sup> B<sub>1</sub> + G<sub>1</sub>) were found in 2 percent of the samples, although 47 percent of the samples exceeded the regulatory limit. Levels of 28–997 µg kg<sup>-1</sup> for AFB<sub>1</sub> and 14–149 µg kg<sup>-1</sup> for AFG<sub>1</sub> were found (Brigido et al., 1995). Besides, in a survey of 321 samples of peanuts and groundnut products, a total of 116 samples (36 percent) showed concentration higher than that permitted by the Brazilian Legislation (Sabino et al., 1999).

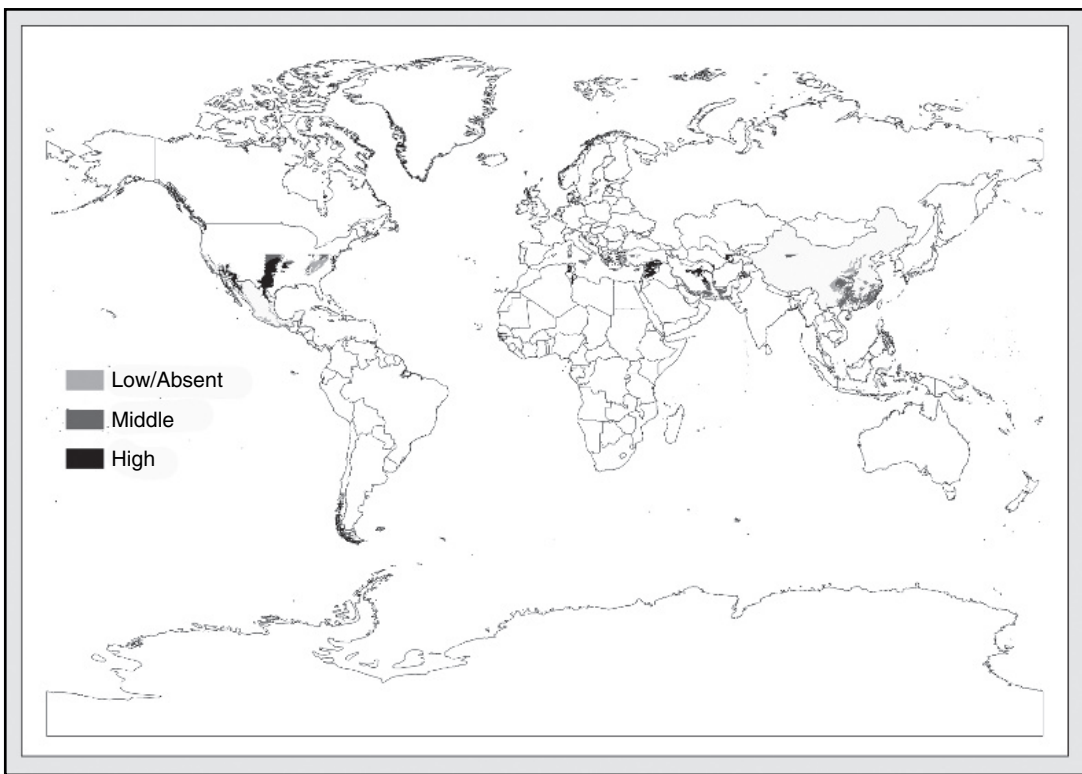
1.3.3. RISK OF AFLATOXIN B<sub>1</sub> CONTAMINATION IN PISTACHIO NUTS

Homogeneous areas were defined also for pistachio nuts, taking into account differences previously underlined in respect to peanuts, using data on temperature and rain, and maps were consequently drawn (Table 1.5).

The total 2005 worldwide production of 501 thousand metric tonnes of pistachio nuts (FAOSTAT) was shared as 38 percent in Iran, 28 percent in the United States, mainly in California, 12 percent in Turkey and in Syria, 7 percent in China, 2 percent in Greece and the remaining 1 percent in the rest of the world. Iran and the neighbouring countries in Europe and Tunisia are described as high-risk areas for AFB<sub>1</sub> contamination of pistachio nuts, while China has a low mean risk. The growing areas in the United States, the most important areas after Iran, are also at high risk for AFB<sub>1</sub>, mainly in California (Fig. 1.3). Surveys on pistachio nuts are not available and these data cannot be confirmed.

1.4. SAFETY EVALUATION OF FRUITS

Relevant mycotoxins are signalled in fruits, with major problems in dried compared to fresh fruits. As a general rule, contamination starts in-field with possible or major increase post-harvest, depending on both the product and the fungus. In-field contamination is mainly related to meteorological conditions and human efforts can only be addressed to the improvement of cropping systems, while driving variables are out of control. Also during the post-harvest period ecological conditions determine the possible increase of



**FIGURE 1.3** Prediction map of aflatoxin B<sub>1</sub> risk in pistachios growing areas on a global scale. The risk was computed with a geostatistical approach using the combination of mean temperature (°C) and summation of rain (mm) during August and February, respectively, in northern and southern hemispheres.

mycotoxin contamination. The possibility of reducing that risk is very high in developed countries and progressively decreases in those places where this part of product management is not done under human control. Drying nuts, for example, can be managed in the open air or in a controlled environment or apple transport and storage can be done in ambient or controlled conditions. Nevertheless, in pre- and post-harvest, products management is optimised when knowledge on all aspects of mycotoxin-producing fungi is complete.

Information on the occurrence of mycotoxins and the fungi that produce them is not the same for all products and certainly not equally accurate for all geographical areas of production. The approach followed in this study tried to find the optimum way to manage and simulate available data. In fact, available meteorological data were chosen to describe the climate in the relevant period for all the crop-fungus systems. Crop growing areas were defined based on their true distribution and general rules were hypothesised and used in the study. Finally, with the help of geostatistics and knowledge of the ecological needs of fungi, the global level of risk was simulated and risk maps were drawn. They take into account meteorology, crop distribution and growing period. It means that the maps shown predict the risk of mycotoxin contamination in the field without yearly meteorological data and the effect of cropping systems.

The approach followed cannot be very precise, because of the global dimension, the simulated approach and the lack of cited data, but surely it represents a good base for evaluation of safety of fruits produced all over the world. This can be considered a prototype approach that can be transferred to other crops and mycotoxins/diseases.

Improvements to the global risk maps could be obtained with more precise information regarding several aspects, like fungal ecology, also in relation to host crop, cropping system or area of crop cultivation. Besides, since the fungi are strictly dependent on yearly meteorological data, better knowledge is obviously related to the availability of yearly data.

Anyway, maps show different high-risk areas all over the world for all mycotoxins considered, mainly AFs, that suggest high attention especially when the country of origin is a third country where fruits are not managed following the best practices.

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# Economic Aspects of Mycotoxins in Fruits and Vegetables

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## ABSTRACT

International trade in fresh fruits and vegetables has grown greatly in the past 20 years and is presently a multi-billion dollar business representing the major export for many developing countries. There has been a global effort to develop and implement safe food handling for the entire food chain at all levels of governments and the food industries. Although many factors can contribute to rejection of fruits and vegetables at the time of importation, the major issue is use of pesticides, both non-permitted pesticides and excessive use of permitted ones. Additional factors responsible for rejection of imports are the presence of contamination by filth, missing mandatory labeling, and lack of required nutritional information. Fruits and vegetables have been known to cause outbreaks of illness in developing countries, but there has been no link to the presence of mycotoxins. No firm evidence exists linking long-term ingestion of mycotoxins in commonly eaten fruits and vegetables with any type of chronic illness. The most well-known economic analysis of the impact of mycotoxins was based on simulated data and has been questioned as to whether the projected impacts were actually borne out by facts. The purpose of this chapter is to examine an important understudied area, the economic aspects of mycotoxins in fruits and vegetables.

## 2.1. INTRODUCTION

All levels of government and food industries have been involved in a global effort to develop and implement safe food handling for the entire food chain. Beneficiaries of these efforts are both a reduction of food safety hazards associated with fresh fruits and vegetables and improvements in trade opportunities.

Three major components are involved in these endeavors: Good Agricultural Practices (GAPs), Good Manufacturing Practices (GMPs), and Hazard Analysis and Critical Control Point (HACCP). All phases of production are covered by GAPs, including the use of land, soil, and water, the control of wildlife and pests, and proper worker hygiene and sanitation. GMPs are based on the same principles as GAPs, with a focus on post-harvest handling: packing, storage, transportation, and handling and processing of fresh produce at the final destination (Gorny, 2006). HACCP is a food management system designed to ensure the safe production of food by identification, evaluation, and control of food safety hazards, based on the following seven principles: (1) Conduct a hazard analysis; (2) Determine the critical control points (CCPs); (3) Establish critical limits; (4) Establish monitoring procedures; (5) Establish corrective actions; (6) Establish verification procedures; and (7) Establish record-keeping and documentation procedures (FSIS/USDA, 1999). The HACCP requirements have been accepted in the United States throughout the food industry. At the present time, many food industry manufacturers are requiring that raw material suppliers have a defined HACCP plan if they wish to supply ingredients and materials to their operations. The US FDA HACCP regulations emphasize prerequisite programs such as Sanitation Standard Operating Procedures (SSOPs), which are designed to control general sanitation issues during production in a facility to prevent contamination or adulteration (FDA and USDA, 2000). When these essential practices are strictly implemented, they ensure the minimization or elimination of food-related hazards.

Many foods can be contaminated with mycotoxins before they are harvested, as well as between harvesting and drying, and in storage. Other than aflatoxin, mycotoxin contamination is poorly studied, although ochratoxin and *Fusarium* mycotoxins have also received attention as food contaminants, the latter of which include deoxynivalenol, zearalenone, and fumonisin can occur in the more temperate regions of the United States and elsewhere. Although a number of mycotoxins can be present in foods imported into the United States, the most important mycotoxin that the US Food and Drug Administration (FDA) regularly tests for is aflatoxin (present US regulatory level in human food, 20 ng/g) (Center for Integrated Fungal Research, 2005). The US FDA produced a regulatory action guidance level for patulin of 50 micrograms per kilogram (50 parts per billion); the compliance level is for the presence of patulin in apple juice, apple juice concentrates and products containing apple juice (FDA, 2001). In addition to apple juice, testing for patulin is performed on apple puree and unfermented cider. For importation into the United States concentrated apple juice must have an accompanying document stating that the juice has been tested for the possible presence of patulin and that patulin is present at a concentration of less than 50 ng/g; recent EU levels have been set at 25 ng/g or 10 ng/g, the latter level for infant foods (Arranz et al., 2005). If patulin is present at concentrations in excess of the advisory level, it is a good indication that poor manufacturing practices have occurred and that moldy material or unclean facilities may have been used. In addition, some monitoring is performed for fumonisin,

deoxynivalenol, and ochratoxin, but these mycotoxins have advisory levels set by FDA, not regulatory levels.

Fruits and vegetables have been known to be associated with outbreaks of illnesses in developing countries, but there has been no link to the presence of mycotoxins. The major contaminants of concern as potentially present in fruits and vegetables are bacteria, viruses, and parasites. Microbial pathogens pose the greatest food safety threat for fresh produce (United Nations, 2007). Analysis is only performed for certain select products likely to contain mycotoxins including aflatoxin in products such as peanuts, corn, grapes, and dates (Jelinek, 1987). There has been no firm evidence to link long-term ingestion of mycotoxins in commonly eaten foods with any type of chronic human illness. Although some mycotoxins have been identified as potent liver carcinogens in experimental animals, if they are present alone, they have not been established as human carcinogens. Chronic exposure to aflatoxin is only a problem if a person is also infected with Hepatitis B (Turner et al., 2002). Mycotoxins are considered chemical hazards, which also include pesticides, antibiotics, and growth hormones (United Nations, 2007). It may be possible to eliminate, or at least minimize, chemical and physical hazards by following GMPs.

## 2.2. TRENDS IN FOOD SAFETY

Food safety standards in QUAD countries (Canada, the European Union, Japan, and the United States) are becoming more stringent. In the United States, no fruit or vegetable variety can be imported unless a Plant Risk Assessment has demonstrated that the importation does not represent an unacceptable risk to the US plant health. Imports of plant products, including fruits and vegetables, into the United States are regulated by the Plant Protection Act (PPA), which became law in June 2000 after 17 years in the making. In the EU, no plant health risk analysis is required to import fruits and vegetables, and restrictive measures are implemented only in cases where a specific problem has been detected. It is expected that food safety standards for entry into the Japanese market will become more stringent, and importers will require additional documentation from suppliers in the future (South Centre, 2005).

In the last 10 years, large supermarket chains in many countries have grown in importance, with wholesale markets either declining or taking on more specialized roles. Imports from specific developing countries have also grown. As hygiene conditions in Kenya have improved, their fresh vegetable exports increased from \$23 million to \$140 million between 1991 and 2003. Another success story is the Peruvian asparagus industry, where adoption of national standards in line with international norms has made Peru one of the world's largest exporters of asparagus. The standards adopted by Peru have included HACCP, traceability systems, and GAP certification. Adoption of these standards enabled many large exporters to be certified under the Euro-Retailer Produce Working Group for Good Agricultural Practices (EUREP-GAP),



which is a stricter protocol than countries previously had (Jaffee, 2003). EUREP-GAP certification is expected to dominate the market in the near future for all producers supplying fresh produce to major European retailers (Willems et al., 2005).

Consumers, at the present time, demand year-round delivery of fresh produce. Food safety control is one of the highest priorities in the fresh produce sector. Retailers are held directly responsible by their customers for the safety of food. Retailers pass this responsibility on to their importers and processors by developing strict food safety and quality protocols. Any recall that may occur affects the importers and retailers, so they visit producers to inspect the quality of the products in the field and to discuss quality and safety issues. In Western Europe, supermarkets account for approximately 80 percent of fruit and vegetable retail sales (Willems et al., 2005). The only quality problem noted for fruits and vegetables from developing countries was spots on the skin of produce indicating fungal presence; mycotoxins were not specifically noted.

Residues of pesticides on fruits and vegetables were noted as representing the major reported food safety problem. The number of reported phytosanitary problems was limited and quality problems occurred more frequently than safety problems (Willems et al., 2005).

The Rapid Alert System for Food and Feed (RASFF) is a system which provides control authorities with an effective tool for exchange of information on measures taken to ensure food safety. At weekly intervals, two different types of notifications (alert and information) are issued. Alert notifications are sent when immediate action is required for food or feed on the market that is presenting a risk. Products subject to an alert notification either have been withdrawn or are in the process of being withdrawn from the market. Issuance of an information notification indicates that no immediate action has to be taken because although a risk has been identified, the product has not reached the markets of other members of the network. These later notifications concern food and feed consignments that have been tested and rejected at the external borders of the EU. For the 51st week of 2006, there were five notifications regarding foods and mycotoxins, of which only two cases involved fruits or vegetables (aflatoxins in dried figs and ochratoxin A in dried raisins), from Turkey and Iran, respectively. The majority of alert notifications involved aflatoxins in nuts. This suggests that mycotoxins are either not present in fruits and vegetables or they are not being detected. The number of notifications concerning third countries should not be compared with those of Member States because the only time that remediation can be carried out on a product from third countries is when it enters the EU community. For products originating within the EU, monitoring is performed throughout the entire food chain, enabling food hazards to be detected at an early stage in the production process. Because the product has not reached the market, hazards detected during production in the EU do not elicit a RASFF notification (European Commission, 2005).

Developing countries, in the past, have claimed that the costs of compliance with sanitary and phytosanitary (SPS) measures they encounter when they

upgrade and maintain HACCP represent an impediment to exports (Henson and Loader, 2001). A large updated body of food and feed legislation for food safety took effect on 1 January 2006 in the EU. It was applied both in the EU and in third countries wishing to export to the EU. Guarantees must be provided by all third countries stating that their products meet the necessary standards required by the EU for importation. Guides to good practice will be established to assist food operators in implementation of their self-checking programs, and all food operators will have to be registered.

From a position of having nearly no apple orchards 20 years ago, China has overtaken the United States as the world's largest apple producer due to low labor costs. China is moving to produce more partially processed products, which are expected to have a higher rate of return on investment. At the same time, as people achieve a higher standard of living, they consume more fruit. In the United States, Mexico, and Canada, fruit and vegetable crops do not benefit from direct government payments, such as price support payments, although a variety of marketing and research programs support the fruit and vegetable industries. For many vegetables, there is little information available on the extent of mycotoxin contamination that occurs. A range of toxic metabolites produced by *Alternaria* species has been detected in infected tomatoes (Hasan, 1996; Bottalico and Logrieco, 1998) and in olives, peppers, and fresh and processed fruits (Scott, 2001). Deoxynivalenol has been detected in potato tubers (El-Banna et al., 1984). Because these reports were not followed by additional studies, there is no widespread concern for their occurrence. There has been worldwide concern, however, for ochratoxin A in wine (Cabañes et al., 2002). *Aspergillus carbonarius* has been cited as being responsible for ochratoxin A contamination of grapes, wine, and vine fruits; in some regions, ochratoxin A has been found in much higher concentrations in dried vine fruit than in wine (Battilani et al., 2006).

Consumption of fresh fruits and vegetables in the United States has increased during the past 30 years. Due to the globalization of the food supply, fruits and vegetables are now available regardless of the season. Produce can now travel long distances with minimal spoilage because of improvements in mass production and distribution. The FDA has worked with foreign governments and industries to improve produce farming and exporting practices thereby preventing contamination of fruits and vegetables. Practices associated with increased risk of contaminated juice include use of fruit that has dropped on the ground and less than adequate cleaning of fruit prior to juicing. Unpasteurized juice has also been linked to outbreaks, but this has been primarily due to bacterial, not mycotoxin, contamination. Methods to detect pathogens on fresh produce are not as developed as they are for poultry and beef products. Effective testing is limited by the sporadic nature of most contamination on fresh produce. Because it is not feasible to eliminate all potential hazards, fruit producers must rely on risk reduction rather than risk elimination (Zhao, 2005). GAPs and GMPs during growing, harvesting, washing, sorting, packing, and transporting fresh fruit serve to minimize microbial food safety hazards. Development of SSOPs and the implementation of HACCP

programs are additional components to help ensure the safety of fresh products. The United States and the United Kingdom are among a small number of countries having a large number of outbreaks concerning fresh produce (European Commission, 2002). This is not due to the fact that they have a particular problem with fresh produce, but rather it is because they have comprehensive surveillance and reporting systems.

### 2.3. NEW SITUATIONS WITH FRUIT AND VEGETABLE EXPORTERS

As the world's largest food importer, Europe has had a strong relationship with Latin America. EU imports approximately 45 percent of the agricultural exports from Latin America, of which 15 percent of exports in 2003 were from Central America (South Centre, 2005). To ensure the safety of its imports, the EU held workshops organized specifically for third countries to ensure safety of EU imports and to provide them with in-depth knowledge of EU import rules for fruits and vegetables. The training focused on ensuring that fruit and vegetable imports from third countries meet EU standards.

The United States imports more than 12 percent or \$58 billion of food from other countries (South Centre, 2005). Approximately 20 percent of fruits and vegetables come from Central and South America. In the past several years, there has been a five-fold increase in the number of food products entering the US market. Training personnel in food safety methods locally in the country of origin is an important component to ensure that foods are produced safely before they are exported. A new food safety training program, the Johnson Diversey International Food Safety Initiative (JDIFSI), was recently proposed to improve the quality of food entering the United States (Food Safety Insider, 2006). This initiative links the Joint Institute for Food Safety and Applied Nutrition (JIFSAN), which was established between the US FDA and the University of Maryland in 1996. It enables the food industry to identify and train local trainers in participating foreign countries on the best practices in food safety. JIFSAN was successful in this approach by conducting educational programs in the countries where the imported products originated, ensuring that foods are safely produced before they are exported.

The presence of China in the global market has risen for fruits and vegetables; the most rapid increase has been in apples, apple juice, and fresh vegetables. Presently, China is the world's leading exporter of apple juice (FAS/USDA, 2005). China's growing demand for fruits and vegetables could rise sharply as they experience income gains, but the supply available for export may be reduced if their domestic consumption of fresh fruits and vegetables rises sharply. Due to phytosanitary issues, Chinese fresh apples are presently not allowed into the United States and Taiwan (Huang and Gale, 2006). As China diversifies its fresh vegetable exports, they are becoming more competitive with US products. Asia is an important market where US produce is facing competition from China. Due to their abundant rural labor supply,

China has a competitive advantage because their low wages and labor costs keep production costs low. On average, developing countries have post-harvest losses of 22 percent. Reportedly, Chinese fruits and vegetables often have high levels of pesticides residues, heavy metals, and other contaminants, although mycotoxins have not been mentioned as a concern (Huang and Gale, 2006). It is not known if mycotoxins are present in Chinese fruits and vegetables, or if an effort has not been made to detect them.

Important chronic health problems may never be detected if a mycotoxin does not first show acute effects. There has to be sufficient evidence that fungal compounds are present in food at the retail level before research funds can be justified to show that the fungi can cause toxic effects and are a significant risk to human health. The presence of fungi capable of producing mycotoxins does not mean that the toxins have been produced; it only means that the fungi are present. Several hundred fungal metabolites can be classified as mycotoxins, but many of them are poorly studied and information about their toxicity is unknown (Carlson and Ensley, 2005). Mycotoxins may be present in animal feeds, but it is not likely that they will be present in human foods from an animal source (CAST, 2003). Exposure to mycotoxins is not typically responsible for acute effects, which are attributed mainly to infections caused by bacteria, viruses, or protozoa. The main concern for human health in developed countries is presently focused on exposure to aflatoxins, ochratoxin A, and fumonisin and is based not on their acute effects, but on their potential carcinogenic, immunological, or genotoxic properties (Dohlman, 2003). It is important to be aware when blaming mycotoxins for causing human diseases that there are statements in the literature that are not backed by scientific evidence (Dohlman, 2004).

Food safety is of major importance within Europe. At the present time, emphasis is placed on minimizing toxins as food contaminants and preventing them from reaching consumers. It is important to remember that the nations most likely to have their food supply exposed to mycotoxin contamination are frequently those having the fewest resources to be able to address the problem. The immediate priority of many people in the developing area of the world is prevention of starvation rather than any long-term illness (Wu, 2004).

In order to determine the economic aspects of mycotoxins in fruits and vegetables, it is necessary to have access to detailed information about the relative cost structure of domestic and foreign industries (Roberts and Krissoff, 2004). Unfortunately, this information is not readily available, thereby making it difficult to determine the net effect of food safety measures on horticultural trade patterns. Exporters in countries that do not require HACCP for domestic production must either invest financially in risk-reduction technology to maintain access to the US and EU markets or divert their products to countries without HACCP requirements (Roberts and Krissoff, 2004). There is no evidence that food imported into the United States is riskier regarding mycotoxins than domestically produced food (Buzby and Mitchell, 2003). The only exception is that contamination by aflatoxin may be present in nuts if they originated from certain areas (Buzby and Mitchell, 2003). Studies and statistics

are primarily available on actual costs only for aflatoxin in high-risk commodities (maize and groundnut). The presence of aflatoxins, the most studied and widely known mycotoxins, was first noted in the early 1960s. Contamination, generally considered a problem in tropical and subtropical regions of Africa, Asia, and Latin America, can also occur in temperate countries of Europe and North America. Prevention of aflatoxin in pistachios is a major concern, but pistachios belong in the category of nuts, not fruits and vegetables (Boutrif, 1998).

Fruits and vegetables do not appear to be of major concern as possible sources of mycotoxin contamination in food and feeds because they were only listed as minor sources in a statement of the Institute of Food Science and Technology Trust Fund (2006). Major sources on the list included mold-damaged foodstuffs, specifically cereals and oilseeds.

Import refusals by the US FDA indicated that the presence of aflatoxins was not a commonly invoked reason for detaining food product imports into the United States in 2001; either contaminated products were detected and diverted to other uses before exportation or the United States did not import a large volume of products (peanuts and corn) most susceptible to aflatoxins (Dohlman, 2003). Contamination by mycotoxins is recognized as an unavoidable risk, yet it is difficult to access economic losses associated with mycotoxin contamination. Rather than regulating the treatment of a commodity during processing, the amount of mycotoxin in the final product is usually set as a tolerance level. No comprehensive analysis of the costs to the US and foreign crop producers is presently available (Roberts and Krissoff, 2004). A key part of the present trade policy debate is how regulatory costs for exporters compare with possible gains in higher SPS levels in importing countries (South Centre, 2005). Empirical information on the trade impact of such standards is not readily available or even known. Based on the precautionary principle, some countries may set new standards for specific mycotoxins, even though there is no scientific evidence of a clear health risk (Dohlman, 2004).

The term of Most-Favored Nation (MFN) is actually a misnomer because it is the least-favored treatment given EU imports (Hasha, 2004). MFN trading partners, including the United States, have their fruit and vegetable exports at a disadvantage in EU markets because they face higher tariffs than countries receiving preferential trading agreements with the EU. Forty-two least-developed countries have the most valuable preferences and an additional 77 former colonies receive important preferences (Hasha, 2004).

Only a small proportion of rejected consignments is actually destroyed at the point of import. Some, perhaps a significant proportion of the product is reshipped, reconditioned, or otherwise managed for sale in the domestic market where the products originated. The FDA only inspects between 1 and 2 percent of the more than 6 million consignments of food and cosmetic products imported each year. An important point to remember is that many aspects of standards compliance do not require large investments or sophisticated technical or administrative capacities (Jaffee, 2005).

Of the developed countries, the European Union was the subject of the largest number of complaints by developing countries. The EU has most frequently and visibly embraced the “precautionary principle” when adopting certain standards, giving rise to controversies over the scientific basis for these measures (Jaffee and Henson, 2004). “We have not effectively examined the economics of mycotoxins in fruits/vegetables as it relates to developing countries” (personal communication, Steven Jaffee). Mycotoxin concerns in developing countries are more commonly examined in relation to cereals, nuts, and alcoholic beverages, usually from a technical or a health perspective, rather than from an economic perspective. The FAO has done a lot of work on mycotoxins in developing countries, yet rarely are economic dimensions examined. In horticultural crops, mycotoxins are primarily associated with dried fruits (figs and prunes), certain processed products (apple and grape juice) and are probably in apples and grapes. South Africa, Chile, Argentina, and Turkey are likely to have developed technical and economic assessments since they produce concentrated apple juice for export. For import into the United States, apple juice concentrate must be labeled to document that it complies with the US advisory level for patulin of 50 ng/g (FDA, 2001). In a recent article, the following statement was made: “We did not find any studies of mycotoxin costs (as they relate to food safety) in the published literature” (Unnevehr and Jensen, 2005).

The main barriers to fresh imports into Japan are phytosanitary to protect against the introduction of diseases that could hurt domestic productions (South Centre, 2005). Certain vegetables (fresh cucumbers, eggplants, potatoes) are banned. Where a disease is known to exist, Japan requires very strict, expensive protocols for farms in foreign regions, including onsite inspection by Japanese authorities (Huang, 2004). Similarly, the United States does not allow the importation of certain products (apples) where introduction of disease is a possibility.

Most retailers have developed their own specifications for imported fresh fruits and vegetables; factors include a range of organoleptic criteria, appearance, grading, ripening, maximum residue limits/levels (MRLs), packing, labeling, and phytosanitary specifications. The majority of food safety problems with products imported from developing countries are related to pesticide residues (South Centre, 2005). When considering factors of importance in food safety and quality, MRLs from pesticides rank higher than the presence of fungi in fruits and vegetables. It is commonly held that quality problems occur more frequently than safety problems.

The US FDA’s voluntary guidelines for GAPs provide recommendations to growers, both domestic and foreign, on how to reduce microbial hazards. The emphasis is to reduce the risk of microbial contamination because it is not possible to eliminate the risk. The FDA cannot rely on random testing at the borders to detect contaminated produce because it is very difficult to identify microbial contamination. Instead of testing, the FDA has concentrated the majority of its efforts on education in foreign countries, although the FDA will do inspections when there is reason to expect a problem (Calvin, 2004).

Because of environmental conditions, some countries are more likely to have specific mycotoxins present in certain crops.

Adverse effects of consuming food contaminated with toxins may not be observed for many years, if ever, and it is almost impossible to link to a particular food, thereby making risk-assessment difficult (Dohlmán, 2003). Firms must adhere to procedures in their HACCP plans, but are allowed to define those procedures. Both the United States and the EU require producers of certain food products (e.g., apple juice) to implement HACCP plans. Regulations may give a cost advantage to large firms if they are able to afford large initial expenditures on equipment and can amortize the cost per unit over a large number of units (Mitchell, 2003). Some retailers prefer to require processors for outside certification, rather than analyzing the samples in-house (Henson and Northern, 1998). Approximately 190 countries of the world have all established different regimes for food safety and thousands of different foods are regulated. The cost to comply with food safety regulations is often prohibitive for poorer countries (Unnevehr, 2000). If firms are allowed to charge a premium in the market having more stringent standards, they may adopt the standards of their trading partners, thereby improving food safety in their own domestic market.

Implementation of the new EU aflatoxin standard may have a negative impact on African exports of cereals, dried fruits, and nuts to Europe. The European Commission's approach has been challenged in trade policy talks on the basis that import restrictions have been imposed without sufficient support of international science (Dohlmán, 2004). In developed countries, aflatoxin contamination rarely occurs at levels that cause notable aflatoxicosis in humans. In the past, it was thought that aflatoxin alone was responsible for aflatoxicosis; current thinking is that aflatoxicosis is caused by the combination of ingestion of aflatoxin and the presence of the hepatitis virus (Turner et al., 2002). The Codex standard on total aflatoxin in processed and unprocessed food is set at 15 ppb. The harmonized EU standard for total aflatoxin contamination in food products is more restrictive (4 ppb). It is clear that the least-developed countries are relatively more vulnerable to regulatory changes than industrialized nations because of scarcity of public resources to finance compliance with SPS standards. Otsuki et al. (2001) suggested that there can be a very high cost to exporters in setting standards to address relatively small risks to human health. Others have cautioned that the estimates of Otsuki et al. (2001) were interpreted as actual losses of trade, rather than the results of a simulation exercise and there were problems with the baseline data, assumptions, and conclusions used in their simulation (Jaffee and Henson, 2004).

Only a few mycotoxins (aflatoxins, ochratoxin A, and patulin) are regularly found in foods (Drusch and Aumann, 2005). Toxins produced by *Fusarium* species may be present, but they are found mainly in grains and seeds. The most effective means for controlling growth of mycotoxigenic fungi on fruits in the field and in storage is application of fungicides which cannot be done when raising organic fruit (Drusch and Aumann, 2005). In Egypt, a wide variety of toxigenic fungi, mainly of the genera *Aspergillus* and *Penicillium*,



have been isolated from strawberries, apricots, plums, peaches, grapes, dates, figs, apples, pears, and mulberries (Aziz and Moussa, 2002). A large number of molds are able to produce patulin, but their occurrence is limited mainly to fruits (apple juice, apples, and apple products). Efficient chemical decontamination strategies for patulin-contaminated foods (apple juice and apple juice concentrate) do not exist (FDA, 2004b).

The most recent forecast for world production of apple juice for market year 2005/06 (July–June) is 1.26 million metric tons (People’s Republic of China, 2006). Nearly 49 percent of the world production of apple juice is expected to be from China (Fig. 2.1).

Juicing companies are presently not using out-of-grade apples for processing because improvement in orchard management has resulted in fewer out-of-grade apples being available (FAS/USDA, 2006). Most of the juice apples are culled fruit from fresh packing lines. More than 90 percent of Concentrated Apple Juice (CAJ) produced in China was exported in 2004, with the United States being the largest importer of Chinese CAJ (by volume and value) (People’s Republic of China, 2004). US imports are mainly concentrated non-frozen apple juice (FAS/USDA, 2005). In the United States only about 16 percent of total apple production goes into the juice and cider market. Both imported and domestic juice in the United States are required to be processed according to the FDA’s Juice HACCP Regulation (21 CFR120). According to the regulation, importers must ensure that the juice they are importing meets those requirements. As of 25 September 2006, 48 juice-processing plants were online in China. These plants have been verified by the Certification and Accreditation Administration of the People’s Republic of China (CNCA) as meeting the FDA’s juice HACCP regulation (People’s Republic of China, 2006). Imported juice meets requirements by several means, either by a continuing or lot-by-lot certificate from an appropriate foreign government

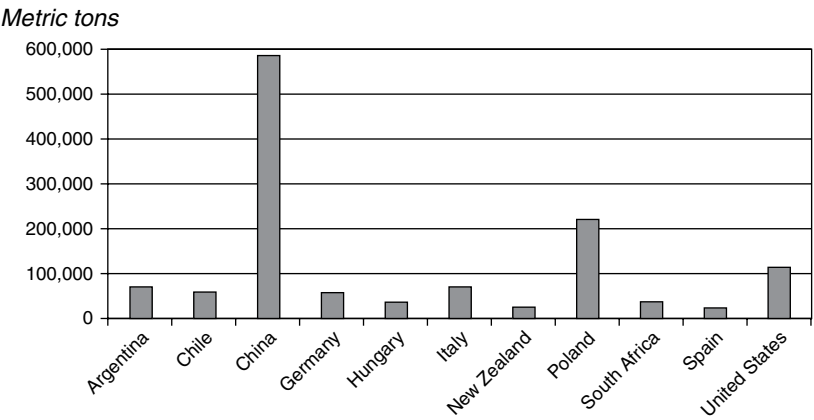


FIGURE 2.1 World Apple Juice Producers in 2005/06. Eleven major countries are shown. Source: Foreign data is based on USDA/FAS Attaché Reports. The US data is based on National Agricultural Statistics Service (NASS) statistics.



inspection authority or from a competent private party certifying that the products were produced in accordance with the US requirements.

Prior to the adoption of HACCP, the regulatory monitoring system was based on observations of processing operations, and collection and analysis of regulatory samples at the time of inspection (Motarjemi and Kaferstein, 1999). Detailed guidance for industry regarding the initiation of HACCP activities has been presented by FDA (FDA, 2004a). Under HACCP, monitoring of imported juice products is done by FDA by inspection of the juice importer's records, not by FDA inspections of foreign processing facilities (Kashtock, 2004).

## 2.4. COMMENTS ON THE CURRENT FOOD SAFETY SYSTEM

A wide assortment of fruits and vegetables is now available worldwide. Developing countries have realized significant growth in exports, increasing from \$8.98 billion in 1981 to \$23.88 billion in 2001; Kenya's vegetable trade with the UK has increased from 55 to 70 percent in the past 10–15 years, and Peru's asparagus is now one of its largest export products (Jaffee and Masakure, 2005). Factors responsible for this progress are adoption of HACCP systems, application of GAP, and development of traceability systems (Jaffee and Masakure, 2005). The market and regulatory context of the fresh produce trade is rapidly changing, especially in relation to meeting the demand of the fresh vegetable markets of the European Union. In Peru, industry leaders and government specialists have worked together to adopt international and national standards, resulting in the Peruvian asparagus industry becoming one of the world's largest exporters of asparagus products.

The emergence of third-party certification (TPC) has become a significant regulatory mechanism in the global agrifood system, reflecting a broader shift from public to private governance with the growing power of supermarkets to regulate the global agrifood system (Hatanaka et al., 2005). As growing numbers of major retailers request certification, TPC may become associated with gaining a competitive advantage and more about simply remaining competitive in the marketplace. An example of TPC is EUREGAP, developed by a consortium of leading supermarket chains. Within a few years, more than 13 000 suppliers in 32 countries have become EUREGAP-certified (Hatanaka et al., 2005).

Even Turkey, a country having the highest rejections by the EU, has been little affected by the increased stringency of the EU's standards (Mencarelli et al., 2005). The volume of products rejected by the EU in 2002 was less than 1 percent of the Turkish exports of nuts and dried fruit. The rejected products probably were either re-exported to countries with less strict standards or enforcements, or sold domestically. In the past 10 years, the produce industry has been transformed by supermarket chains gaining in importance while wholesale markets have declined in importance (Dolan and Humphrey, 2000). As a result, there has been a consolidation among importers, packers, and distributors with the fresh produce market now being managed by fewer players.

In 2002, the EU alerted European countries to import dry fruit from Turkey only if the batches were guaranteed by certified analyses for mycotoxins (Mencarelli et al., 2005). It is impossible to determine accurately the economic costs of mycotoxins. If mycotoxins are present in plants, they can result in lower yields and produce crops having lower value; there are also the costs of affects on animal production (Mencarelli et al., 2005). Direct losses of greater than \$100 million were the result of a *Fusarium* epidemic in Ontario, Canada, in the winter of 1996. Costs due to the affects on animal production, and long-term health effects on humans have not been added to this loss estimate (Warriner, 2005). Losses from mycotoxins in the United States and other industrial nations are typically associated market losses, as opposed to illnesses or deaths from the effects of the toxins, whereas in the developing world the economic and health impacts of mycotoxins are far more serious (Wu, 2004).

Although a large number of different mycotoxins exist, there are only a few of them that are regularly found in foods. Most reports concerning aflatoxin formation on fruits refer to figs or citrus fruits (Drusch and Ragab, 2003). Aflatoxins constitute a problem that is already present in the orchard. Unfortunately, there are no methods for decontamination of food products. Little contamination occurs when firm, ripe fruits are dried immediately (Steiner et al., 1988). From a practical point of view, the best approach for eliminating mycotoxins from foods is to prevent mold growth at all levels of production, including harvesting, transport, and storage (Boutrif, 1998). The most important benefits of food safety regulations are those arising from improvements in human health, including reduced medical costs, fewer productivity losses, reduced risk of foodborne illness, food safety, and improved nutrition. Costs resulting from regulation include companies changing production processes and making investments regarding each unit produced. HACCP was mandated by federal regulation in 2001, but specific HACCP plans are not mandated because the specific industries formulate their own plans (Unnevehr and Jensen, 2005). Presently, both international and domestic suppliers of produce to major US supermarket chains must certify food safety practices (Calvin and Cook, 2001).

Spoilage of fruits is distinct and separate from safety considerations. Spoilage of most fruits is caused by fungal growth, not mycotoxin formation. Routine microbiological testing of fruit is not recommended. There are only certain mycotoxins that are associated with being potentially present in specific products. Patulin and ochratoxin A (OA) are more of a problem in juice manufacture; *Byssochlamys nivea*, in canned fruit products. No mycotoxins are found in fermented juice products. Patulin in apples and pears (*P. expansum*) and OA in grapes (*A. carbonarius*) are the only mycotoxins currently considered to be important in fruit products. The presence of patulin in juice is considered as evidence that unsound fruit has been used in juice manufacture.

The EU is the world's largest importer of fruits and vegetables with marketing standards currently applied to 36 different fruits and vegetables (Elinder,

2003). Minimal requirements are established for cleanliness of the products, absence of pests and damage caused by pests, and freedom from foreign smell and odor. Fruits and vegetables must be sufficiently mature to complete their maturity process and yet be able to withstand handling and transport.

## 2.5. CONCLUSIONS

On a global scale, the mycotoxins of major importance include aflatoxins in tree nuts, dry fruits, and spices, *Fusarium* toxins in cereals (mainly maize, wheat, and barley), and ochratoxin A in cereals and coffee. On a regional basis, mycotoxins of importance include primarily patulin in fruits, ochratoxin A in grapes and dried vine fruits, and aflatoxins in dried fruits. Environmental conditions (climate) strongly influence fungal growth. The US FDA proposed in June 2004 an Action Plan to minimize foodborne illness associated with fresh produce consumption having four general objectives: (1) Prevent contamination of fresh produce with pathogens; (2) Minimize the public health impact when contamination occurs; (3) Improve communication with producers, packers, processors, transporters, distributors, preparers, consumers, and other government entities about fresh produce; and (4) Facilitate and support research relevant to the contamination of fresh produce. Fruit producers and processors must rely on risk reduction rather than risk elimination because it is not currently feasible to eliminate all potential hazards associated with fresh produce. In 2001, the FDA had published final regulations mandating the application of HACCP principles to the processing of juice. Except for food exporting companies, the application of HACCP has made relatively little progress in the developing countries (Motarjemi and Kaferstein, 1999).

The Centers for Disease Control and Prevention (CDC) estimates that foodborne diseases cause approximately 76 million illnesses, 325 000 hospitalizations and 5000 deaths in the United States each year (Mead et al., 1999). The estimated economic cost of US foodborne illness ranges from \$10 to \$83 billion each year. Food illness is associated with a range of foods, including fresh produce. The fact that produce is often consumed raw without any type of intervention to reduce, control, or eliminate pathogens prior to consumption contributes to its potential as a source of foodborne illness. An important point to keep in mind is that the majority of foodborne illnesses are due to bacteria, viruses, parasites, and protozoa (FDA, 2004b). It is impossible to determine accurately the economic costs of mycotoxins. As growing numbers of major retailers are requesting certification, TPC may become less about gaining a competitive advantage and more about simply remaining competitive in the marketplace. In the past 10 years, the produce industry has been transformed by supermarket chains gaining in importance while wholesale markets have declined in importance. As a result, there has been a consolidation among importers, packers, and distributors with the fresh produce market now being managed by fewer players.

## ACKNOWLEDGMENTS

Special thanks are given to Dr Hoonae Kim and Dr Steven M. Jaffee of The World Bank and to Dr Laurian J. Unnevehr of the University of Illinois at Urbana-Champaign for helpful discussions and valuable information on the topic and to Dr Heather Velthuis of USDA/Foreign Agricultural Service for access to the chart entitled “World Apple Juice Producers 2005/06.”

Names of equipment and chemical supplies are necessary to report factually on experimental methods; however, the USDA neither guarantees nor warrants the standard of the products, and the use of the name by USDA implies no approval of the products to the exclusion of others that may also be suitable.

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# Regulations and Limits for Mycotoxins in Fruits and Vegetables

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## ABSTRACT

Regulations on mycotoxins have been established in many countries to protect the consumer from the harmful effects of mycotoxins. Current regulations mostly concern the aflatoxins, but regulations for other mycotoxins are now also rapidly developing. Various factors play a role in the decision-making process of setting limits for mycotoxins. These include scientific factors such as the availability of toxicological data and survey data, knowledge about the distribution of mycotoxins in commodities, and analytical methodology. Economical and political factors such as commercial interests and sufficiency of food supply have their impact as well. By the end of 2003, about 100 countries (covering approximately 85 percent of the world's inhabitants) had specific regulations or detailed guidelines for mycotoxins in food.

Many countries have specific regulations and limits for mycotoxins in fruits and vegetables. In particular, they have been established for the categories nuts, herbs and spices, fruits and vegetables *senso stricto*, as well as for products that may contain fruits and vegetables, such as baby foods and dietary products. Most of the regulations concern aflatoxins (particularly for nuts) as well as ochratoxin A and patulin (both particularly for fruits and vegetables). The aflatoxins and ochratoxin A cause most of the mycotoxin rapid alerts in the EU's Rapid Alert System for Food and Feed. Patulin is of much lesser concern. Peculiar mycotoxins regulated in fruits and vegetables include zearalenone in nuts, phomopsins in lupin seeds, agaric acid in food containing mushrooms and aflatoxin M<sub>1</sub> in fruits and vegetables.

## 3.1. INTRODUCTION

All nations have the right and the duty to protect their citizens from the harmful effects that undesirable substances in food may cause. Among these



compounds, mycotoxins are subject to regulations in many countries, and specific regulations have been established to limit their presence in food and animal feed since the late 1960s. In 2004 approximately 100 countries were known that had established specific limits for various combinations of mycotoxins and commodities, often accompanied by prescribed or recommended procedures for sampling and analysis (Food and Agriculture Organization, 2004). In this article the factors influencing the constitution of mycotoxin regulations will be reviewed. Some general observations are presented about the current situation with respect to worldwide regulations for mycotoxins in food. The focus will then be particularly directed on the mycotoxin regulatory situation with respect to fruits and vegetables. Finally some conclusions will be made.

## 3.2. FACTORS INFLUENCING THE CONSTITUTION OF MYCOTOXIN REGULATIONS

Several factors may influence the establishment of mycotoxin limits and regulations. These include

- availability of toxicological data of mycotoxins
- availability of exposure data of mycotoxins
- knowledge of the distribution of mycotoxins concentrations within a lot
- availability of analytical methods
- legislation in other countries with which trade contacts exist
- need for sufficient food supply.

The first two factors provide the necessary information for hazard assessment and exposure assessment respectively, the main ingredients for risk assessment. Risk assessment is the scientific evaluation of the probability of occurrence of known or potential adverse health effects, resulting from human exposure to food-borne hazards. It is the primary scientific basis for the constitution of regulations. The third and fourth factors are important elements to make practical enforcement of the mycotoxin regulations possible. The last two factors are of socio-economic nature, but these are equally important in the decision-making process to establish meaningful regulations and limits for mycotoxins in foods and feeds.

### 3.2.1. RISK ASSESSMENT

Regulations are primarily made on the basis of known toxic effects. For the mycotoxins currently considered most significant (aflatoxins, ochratoxin A, patulin, fumonisins, zearalenone and some of the trichothecenes, including deoxynivalenol), the Joint Expert Committee on Food Additives (JECFA), a scientific advisory body of the World Health Organization (WHO) and the Food and Agriculture Organization (FAO), has evaluated their hazard in several sessions (World Health Organization, 1990, 1991, 1996, 1999, 2000,

2001). In addition to information about toxicity, exposure assessment is another main ingredient of the risk assessment. Reliable data on the occurrence of mycotoxins in various commodities and data on food intake are needed for exposure assessment. The quantitative evaluation of the intake of mycotoxins is quite difficult. In its 56th meeting, JECFA stressed the importance of the use of validated analytical methods and the application of analytical quality assurance (see also the section on sampling and analysis procedures) to ensure that the results of surveys provide a reliable assessment of intake (World Health Organization, 2002).

In many countries activities take place that contribute to the risk assessment process of mycotoxins. In the EU, for example, several activities take place that contribute to the risk assessment process of mycotoxins. An important role is played by the European Food Safety Authority (EFSA). EFSA is an independent body of the European Commission, established in 2002, and, among other tasks, charged with the development of risk assessments on issues of concern in the food and feed supply. At the time of writing this article, EFSA was re-evaluating the risk of aflatoxins in tree nuts. Depending on the outcome, this might lead to a change (probably an increase) of the EU regulatory limits for aflatoxins in tree nuts. Another important EU activity is SCOOP (Scientific Cooperation on Questions relating to Food), funded by the European Commission, and targeted to make the best estimates of intake of contaminants by EU inhabitants. In the 1990s, these activities resulted in a report on the exposure assessment of aflatoxins (European Commission, 1997). SCOOP reports were later published for several other mycotoxins including ochratoxin A (Miraglia and Brera, 2002), patulin (Majerus and Kapp, 2002) and several *Fusarium* toxins: trichothecenes, fumonisins and zearalenone (Gareis et al., 2003).

### 3.2.2. SAMPLING AND ANALYSIS

The distribution of the concentration of mycotoxins in products is an important factor to be considered in establishing regulatory sampling criteria. The distribution can be very heterogeneous, as is the case with aflatoxins in peanuts and figs. The number of contaminated peanut kernels in a lot is usually very low, but the contamination level within a kernel can be very high. If insufficient care is taken for representative sampling, the mycotoxin concentration in an inspected lot may therefore be wrongly estimated. Similar situations could occur with other mycotoxin/commodity combinations. The risk to both consumer and producer must be considered when establishing sampling criteria for products in which mycotoxins are heterogeneously distributed. Several countries around the world gradually established detailed science-based sampling plans, which take account of the heterogeneous distribution of mycotoxins in agricultural commodities. Examples of official sampling plans for mycotoxins are those applied by the United States (Food and Drug Administration, 2002) and by the EU for several mycotoxins (Commission of the European Communities, 2006a).

Legislation calls for methods of control. Reliable analytical methods will have to be available to allow enforcement of the regulations in daily practice. In addition to reliability, simplicity is desired, as it will influence the amount of data that will be generated and the practicality of the ultimate measures taken. The reliability of mycotoxin analysis data can be improved through the use of interlaboratory-validated methods of analysis (e.g., methods of AOAC International and methods, standardized by CEN [Comité Européen de Normalisation]: the European equivalent of ISO). The use of good, validated methods of analysis is no guarantee to obtain reliable analytical results in mycotoxin determination. Very important, especially in free trade areas, is how enforcement bodies handle an issue as measurement uncertainty. European legislation for mycotoxins requires that 'The analytical result must be reported as  $x \pm U$  whereby  $x$  is the analytical result and  $U$  is the expanded measurement uncertainty' (Commission of the European Communities, 2006a; Stroka and Van Egmond, 2006), but detailed guidance on how to estimate measurement uncertainty is not yet provided. CEN has been mandated by the EC to prepare a new document on criteria for standardized methods to determine mycotoxins in the coming years, which will include discussion of measurement uncertainty.

### 3.2.3. TRADE CONTACTS AND FOOD SUPPLY

Preferably, regulations should be brought into harmony with those in force in other countries with which trade contacts exist. In fact, this approach has been applied in the areas of the EU, MERCOSUR and Australia/New Zealand, where now harmonized regulations for some mycotoxins exist. Strict regulative actions may lead importing countries to ban or limit the import of commodities, which can cause difficulties for exporting countries in finding or maintaining markets for their products. The distortion of the market caused by mycotoxin regulations in importing countries could lead to export of the less contaminated foods and feeds leaving those inferior foods and feeds for the local market, and to severe financial losses for exporting countries (Wilson and Otsuki, 2001). Later data indicated, however, that border rejections did not necessarily affect the economic return for the developing countries (World Bank, 2005).

The regulatory philosophy should not jeopardize the availability of some basic commodities at reasonable prices. Especially in the developing countries, where food supplies are already limited, drastic legal measures may lead to lack of food and to excessive prices.

### 3.2.4. SYNOPSIS

Weighing the various factors at the interface of science, food security and regulations is not a trivial activity and common sense is a major factor for reaching a decision. Public health officials are confronted with a complex

problem: mycotoxins, and particularly the carcinogenic mycotoxins, should be excluded from food as much as possible. Since the substances are present in foods as natural contaminants, however, human exposure cannot be completely prevented, and exposure of the population to some level of mycotoxins has to be tolerated. Despite the dilemmas, mycotoxin regulations have been established in the past decades in many countries, and newer regulations are still being drafted. Particularly in the European Union many developments take place in the mycotoxin regulatory area, which are scientifically underpinned by various European activities.

### 3.3. THE MYCOTOXIN REGULATORY SITUATION

#### 3.3.1. WORLDWIDE MYCOTOXIN REGULATIONS

Several times in the last decades (in 1981, 1987, 1995, 2003), international inquiries were held and published about regulations for mycotoxins in food and feed (Schuller et al., 1983; Van Egmond, 1989; Food and Agriculture Organization, 1997; Food and Agriculture Organization, 2004). The most recent enquiry in 2003 was carried out by the National Institute for Public Health and the Environment, under contract with FAO. The results of the enquiry were published in detail by FAO in an FAO Food and Nutrition Paper (Food and Agriculture Organization, 2004). On a worldwide basis, at least 99 countries had mycotoxin regulations for food and/or feed in 2003. For several mycotoxins, specific regulations existed for the naturally occurring aflatoxins and aflatoxin M<sub>1</sub>; the trichothecenes deoxynivalenol, diacetoxyscirpenol, T-2 toxin and HT-2 toxin; the fumonisins B<sub>1</sub>, B<sub>2</sub> and B<sub>3</sub>; agaric acid; the ergot alkaloids; ochratoxin A; patulin; phomopsins; sterigmatocystin; and zearalenone. Most of these regulated mycotoxins are produced by *Aspergillus*, *Penicillium* and *Fusarium* species. Whereas *Alternaria* species are among the major fungi in fruits and vegetables (see Chapter 8), no country in the world has formal regulations and limits for any of the *Alternaria* toxins.

#### 3.3.2. REGULATIONS FOR FRUITS AND VEGETABLES

For the purpose of this chapter, the authors have used FAO Food and Nutrition Paper 81 to make an excerpt of those mycotoxin regulations that directly or indirectly apply for fruits and vegetables. This category of food is widely defined, so as to include nuts and processed foodstuffs, such as juices, nectars, baby food and other fruit-derived products, as well as spices, many of which originate from vegetables. The category 'all foods' was also considered relevant, as it could contain fruits and vegetables. This resulted in Table 3.1 which provides detailed insight in the mycotoxin regulations that existed worldwide for fruits and vegetables in late 2003. There are 93 countries with regulations

TABLE 3.1 Maximum tolerated levels for mycotoxins in fruits and vegetables.

Country	Commodity	(Sum of) Mycotoxin(s)	Limit (µg/kg)	Remarks
	Mycotoxins	Abbreviations used here	CAS Registry Number	
	aflatoxin B <sub>1</sub>	afla B <sub>1</sub>	1162–65–8	
	aflatoxin B <sub>2</sub>		7220–81–7	
	aflatoxin G <sub>1</sub>		1165–39–5	
	aflatoxin G <sub>2</sub>		7241–98–7	
	aflatoxins B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>		
	aflatoxin M <sub>1</sub>	afla M <sub>1</sub>	6795–23–9	
	aflatoxin M <sub>2</sub>	afla M <sub>2</sub>	6885–57–0	
	agaric acid		666–99–9	
	deoxynivalenol	DON	51481–10–8	
	ochratoxin A		303–47–9	
	patulin		149–29–1	
	phomopsis A		64925–80–0	
	T-2 toxin		21259–20–1	
	zearalenone		17924–92–4	
ALGERIA [DZ] 2003				
	peanuts, nuts	afla B <sub>1</sub>	10	
		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	20	
ARGENTINA [AR] 2003 [MERCOSUR member state]				
	regulations of Argentina in addition to MERCOSUR harmonized regulations:			
	peanuts exported to EU: see EU			
ARMENIA [AM] 2003				
	all foods	afla B <sub>1</sub>	5	
		zearalenone	1000	
		T-2 toxin	100	
	tomato paste, apple	patulin	5	

AUSTRALIA [AU] 2003	all regulations harmonized with New Zealand			
	peanuts, tree nuts	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	15	
	lupin seeds and products thereof	phomopsins	5	
	food containing mushrooms; alcoholic beverages	agaric acid	100000	
AUSTRIA [AT] 2003 [EU member state]	regulations of Austria in addition to EU harmonized regulations:			
	other products [outside EU regulations]	afla B <sub>1</sub>	1	since 1986
		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	5	
BARBADOS [BB] 2003: situation 1991 [FAO 1997]	all foods	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	20	
BELIZE [BZ] 2003: situation 1991 [FAO 1997]	groundnut	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	20	
BELARUS [BY] 2003	leguminous plants	afla B <sub>1</sub>	5	
	infant food		not allowed	
	mushrooms, fruits, vegetables	patulin	50	
	infant food	DON	not allowed	
	infant food	zearalenone	not allowed	
	infant food	T-2 toxin	not allowed	
BOSNIA [BA] 2003: situation 1981 [FAO 1997]	beans	afla B <sub>1</sub> G <sub>1</sub>	5	
BRAZIL [BR] 2003 [MERCOSUR member state]	regulations of Brazil in addition to			
	MERCOSUR harmonized regulations:			
	all foodstuffs	afla B <sub>1</sub> G <sub>1</sub>	30	

(Continued)

TABLE 3.1—(Cont'd)

Country	Commodity	(Sum of) Mycotoxin(s)	Limit (µg/kg)	Remarks
CANADA [CA] 2003				
	nuts and nut products	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	15	calculated on the nut meat portion; in force since 1969
	sofin, flaxseed, canola, soybeans, mustard seed, peas, canada pea beans, canada lentils	ergot <sup>a</sup>	not applicable	various tolerances exist, expressed as % by weight; <sup>a</sup> see footnote
CHILE [CL] 2003				
	all foods	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub> zearalenone	5 200	
CHINA [CN] 2003				
	peanut and peanut products, peanut oil, irradiated peanut	afla B <sub>1</sub>	20	
	edible vegetable oil		10	
	soya bean sauce, beans, fermented foods, fermented bean products, fermented wine, salad oil		5	
	infant formula-soybean based, infant formula '5410', formulated weaning foods (soybean based), weaning supplementary foods (soybean among other components)		non-detectable	infant formula '5410' not specified
	semi-finished products (juice or paste)	patulin	100	
	fruit juice or jam, fruit wine, canned products, hawthorn strip (cake)		50	
CODEX ALIMENTARIUS 2003	[international FAO/WHO organisation with 171 member countries, deriving maximum limits (standards) for additives and contaminants in food that are decisive in trade conflicts; CODEX standards are not binding on governments]			
	peanuts, raw	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	15	
	apple juice and apple ingredients in other beverages	patulin	50	

COLOMBIA [CO] 2003		
all foods	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub> afla M <sub>1</sub> afla M <sub>2</sub>	10
CROATIA [HR] 2003 (EU candidate member state)		
beans, peanuts	afla B <sub>1</sub>	5
spices		30
almonds, hazelnuts, walnuts	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	3
juice and concentrates, apples	patulin	50
CUBA [CU] 2003		
peanuts	afla B <sub>1</sub>	5
all foods	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	5
fruits	patulin	50
DOMINICAN REPUBLIC [DO] 2003: situation 1991 [FAO 1997]		
groundnut, soya, tomato(products)	afla B <sub>1</sub> G <sub>1</sub>	0
EGYPT [EG] 2003		
peanuts	afla B <sub>1</sub> afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	5 10
EUROPEAN UNION [EU] 2006		
EU member states: Austria, Belgium, Bulgaria, Czech Republic, Cyprus, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Poland, The Netherlands, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, United Kingdom		
EU EFTA countries: Norway, Iceland, Liechtenstein following EU harmonized regulations (exclusive Switzerland with their own regulations)		
groundnuts, nuts and dried fruit and processed	afla B <sub>1</sub>	2 A
products thereof, intended for direct human	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	4
consumption or as an ingredient in foodstuffs		
groundnuts to be subjected to sorting, or other	afla B <sub>1</sub>	8
physical treatment, before human consumption or	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	15
use as an ingredient in foodstuffs		

(Continued)



TABLE 3.1—(Cont'd)

Country	Commodity	(Sum of) Mycotoxin(s)	Limit (µg/kg)	Remarks
	nuts and dried fruit to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	afla B <sub>1</sub> afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	5 10	
	spices: <i>Capsicum spp.</i> (dried fruits thereof, whole or ground, including chillies, chilli powder, cayenne and paprika); <i>Piper spp.</i> (fruits thereof, including white and black pepper); <i>Myristica fragrans</i> (nutmeg); <i>Zingiber officinale</i> (ginger); <i>Curcuma longa</i>	afla B <sub>1</sub> afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	5 10	
	dietary foods for special medical purposes intended specifically for infants	afla B <sub>1</sub>	0.10	C
	dried vine fruit (currants, raisins and sultanas)	ochratoxin A	10	D
	dietary foods for special medical purposes intended specifically for infants	ochratoxin A	0.50	F
	fruit juices and fruit nectar, in particular apple juice, and fruit juice ingredients in other beverages	patulin	50	G
	concentrated fruit juice after reconstitution as instructed by the manufacturer		50	
	spirit drinks, cider and other fermented drinks derived from apples or containing apple juice		50	
	solid apple products, including apple compote, apple puree intended for direct consumption		25	
	apple juice and solid apple products, including apple compote and apple puree, for infants and young children and labelled and sold as intended for infants and young children		10	
	other baby foods (as defined in Article 1 of [EUa]) other than processed cereal-based foods		10	

FINLAND [FI] 2003 [EU member state] regulations of Finland in addition to EU harmonized regulations:	all spices	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	10	guide value until regulation will be approved
	other food products		5	
FRANCE [FR] 2003 [EU member state] regulations of France in addition of EU harmonized regulations:	vegetable oils	zearalenone	200	
GERMANY [DE] 2003 [EU member state] regulations of Germany in addition of EU harmonized regulations:	food for infants and young children	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>3</sub>	0.05	
GUATEMALA [GT] 2003: situation 1991 [FAO 1997] kidney beans, groundnuts, groundnut butter		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	20	
HONDURAS [HN] 2003: situation 1991 [FAO 1997 ref.1] all foods baby food		afla B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	1	
		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	0.01	
		afla M <sub>1</sub>	0.02	
HONG KONG [HK] 2003 foods		afla B <sub>1</sub>	15	
		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	15	
		afla M <sub>1</sub>	15	
	peanuts, peanut products	afla B <sub>1</sub>	20	
		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	20	
		afla M <sub>1</sub>	20	

(Continued)

TABLE 3.1—(Cont'd)

Country	Commodity	(Sum of) Mycotoxin(s)	Limit (µg/kg)	Remarks
INDIA [IN] 2003	all food products	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub> & afla M <sub>1</sub>	30	
INDONESIA [ID] 2003	peanuts, coco nuts, spices, traditional drugs/medicines/herbs	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	20	
IRAN [IR] 2003	pistachio nuts, peanuts, walnuts, other nuts and edible seeds	afla B <sub>1</sub> afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	5 15	The Ministry of Health and Medical Education is responsible for the control of aflatoxins in pistachios, exported to the EU, as well as foodstuffs for the Iranian market. Official sampling plans and methods of analysis exist for aflatoxins in pistachio.
	dates, dried grapes (raisins and sultanas), figs and all dried fruits	afla B <sub>1</sub> afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	5 15	
	fruit juices, nectarine and fruit drinks	ochratoxin A patulin	10 50	
	baby instant food (ready to use)	ochratoxin A	1	
	legumes	afla B <sub>1</sub> afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	5 10	
		ochratoxin A	20	

ISRAEL [IL] 2003	nuts, peanuts, figs and their products and other foods	afla B <sub>1</sub>	5
		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	15
	apple juice	patulin	50
ITALY [IT] 2003 [EU member state]	regulations of Italy in addition to EU harmonized regulations:		
	fruit juice	ochratoxin A	50
	baby food	afla M <sub>1</sub>	0.01
		ochratoxin A	0.5
		zearalenone	20
JAMAICA [JM] 2003: situation 1991 [FAO 1997]	food		
		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	20
JAPAN [JP] 2003	all foods	afla B <sub>1</sub>	10
	apple juice	patulin	50
JORDAN [JO] 2003: situation 1981 [FAO 1997]	almonds, peanuts, pistachio nuts, pine nuts	afla B <sub>1</sub>	15
		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	30
KENYA [KE] 2003: situation 1981 [FAO 1997]	peanut(product)s, vegetable oils		
		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	20
KOREA, REP. [KR] 2003	soy-bean, peanuts, nuts and the products made from these by simple processing such as grinding and cutting	afla B <sub>1</sub>	10
	apple juice, apple juice concentrate	patulin	50
KUWAIT [KW] 2003	infant and children food		
		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	0.05

(Continued)

TABLE 3.1—(Cont'd)

Country	Commodity	(Sum of) Mycotoxin(s)	Limit (µg/kg)	Remarks
MACEDONIA [MK] 2003 (EU candidate member state): situation 1981 [FAO 1997]	beans	afla B <sub>1</sub> G <sub>1</sub>	5	
MALAWI [MW] 2003: situation 1987 [FAO 1997]	peanuts (export)	afla B <sub>1</sub>	5	
MALAYSIA [MY] 2003: situation 1987 [FAO 1997]	all foods	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	35	
MAURITIUS [MU] 2003: situation 1987 [FAO 1997]	all foods	afla B <sub>1</sub>	5	
		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub> M <sub>1</sub> M <sub>2</sub>	10	
	groundnuts	afla B <sub>1</sub>	5	
		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub> M <sub>1</sub> M <sub>2</sub>	15	
MERCOSUR [ME] 2003 (member states 2006: Argentina, Brazil, Paraguay, Uruguay and Venezuela)	peanuts and products thereof	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	20	
MOLDOVA [MD] 2003	legumes, nuts, sunflower	afla B <sub>1</sub>	5	
	juices, canned vegetables, fruits	patulin	50	
MOROCCO [MA] 2003	all foods	afla B <sub>1</sub>	10	proposed legislation
	peanuts, pistache nuts, almonds,		1	
	vegetable oils		5	
	apple juice (products)	patulin	50	
MOZAMBIQUE [MZ] 2003	peanut, peanut milk	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	10	
NEW ZEALAND [NZ] 2003	all regulations harmonized with Australia			

NIGERIA [NG] 2003 foodstuffs	afla B <sub>1</sub>	20	new limits are yet to be established when surveys and stakeholders meetings are held with the assistance of data acquired from a new laboratory that is currently being built in NAFDAC
OMAN [OM] 2003: situation 1987 [FAO 1997] complete foodstuffs	afla B <sub>1</sub>	10	Maximum content referred to a moisture content of 12%
PERU [PE] 2003 raw and processed peanuts	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	15	following Codex guideline limits
PHILIPPINES [PH] 2003 nut (products)	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	20	
RUSSIAN FEDERATION [RU] 2003 grain legumes, including pea, bean, lentil, soya sugary confectionary products nuts oil seeds (sunflower, soya, flax, mustard, rape, peanut) vegetable oils (all kinds) derivatives of vegetable oils (margarine, culinary fat, confectionary fat, mayonnaise, phosphatide concentrates)	afla B <sub>1</sub>	5	

(Continued)

TABLE 3.1—(Cont'd)

Country	Commodity	(Sum of) Mycotoxin(s)	Limit (µg/kg)	Remarks
	isolates, concentrates & hydrolysates of vegetable proteins; flour and coarse meal of pulses, oil seeds and non-traditional food products			
	bottled, canned or potted vegetables, fruit, berries; juices, beverages or concentrates of vegetables, berries (canned); jams, confitures, syrups, fruit & berries mashed with sugar, fruit or berry concentrates with sugar	patulin	50	
SALVADOR, EL [SV] 2003: situation 1991 [FAO 1997]	foods	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	20	
SAUDI ARABIA [SA] 2003	infant and children food	all afla	0.05	SA1
SINGAPORE [SG] 2003	nuts	afla B <sub>1</sub>	not given	
		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	5	
	apple & apple juice	patulin	50	
SOUTH AFRICA [ZA] 2003	all foodstuffs	afla B <sub>1</sub>	5	in force since 1990
		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	10	in force since 1990; a draft is currently in process to increase the limit for aflatoxins in peanuts intended for further processing to 15 µg/kg, to bring it in line with the CODEX level
		patulin	50	in force since 1995

SRI LANKA [LK] 2003	all foods	all afla	30	random sampling
	food for children up to 3 years		1	
SUDAN [SD] 2003	oil seeds	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	see CODEX	
SURINAME [SR] 2003: situation 1991 [FAO 1997 ref.1]	groundnut(products), legumes	afla B <sub>1</sub>	5	
SWEDEN [SE] 2003 [EU member state]	regulations of Sweden in addition to EU harmonized regulations:			
	all foods, not specifically regulated at EU level	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	5	updated in 2002
SWITZERLAND [CH] 2003	nutmeg	afla B <sub>1</sub>	10	
		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	20	
	spices	afla B <sub>1</sub>	5	excluding nutmeg
		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	10	
		ochratoxin A	20	referred to dry matter
	dried fruit	ochratoxin A	20	referred to dry matter
	infant formulae and follow-on formulae; processed	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	0.01	based on ready-to-eat
	baby foods for infants and young children	afla M <sub>1</sub>	0.02	preparation
		ochratoxin A	0.5	referred to dry matter
	all foodstuffs	afla B <sub>1</sub>	2	except those with special
		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	4	regulations
		ochratoxin A	5	
	fruit juices	patulin	50	
SYRIAN ARABIC REPUBLIC [SY] 2003	peanuts and pistachios	afla B <sub>1</sub>	5	
	baby food	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	0.05	
	pulses, mixed nuts, oil seeds and products thereof		20	

(Continued)



TABLE 3.1—(Cont'd)

Country	Commodity	(Sum of) Mycotoxin(s)	Limit (µg/kg)	Remarks
TAIWAN [TW] 2003	peanut	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	15	since 1997
	legumes, nuts		10	
	edible oils and fats		10	
	other foods		10	
	infant food		not detectable	
	apple juice	patulin		
TANZANIA [TZ] 2003	oil seeds	afla B <sub>1</sub>	5	since 1989
		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	10	
THAILAND [TH] 2003	all food products	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	20	In addition, an amendment of the national regulations of mycotoxins is made, by using CODEX maximum levels
TUNISIA (Republic of) [TN] 2003	all products	afla B <sub>1</sub>	2	
		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	unknown	
TURKEY [TR] 2003 [EU candidate member state]	hazelnut, peanut and other nuts; oily seed; dried fruits (fig, raisin, etc.) and foodstuffs produced of these	afla B <sub>1</sub>	5	in force since 2002, but existing since 1990 for all foods
		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	10	in force since 2002, but existing since 1997 for all foods

UKRAINE [UA] 2003	spices	afla B <sub>1</sub>	5	in force since 2002, but existing since 1990 for all foods
		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	10	in force since 2002, but existing since 1997 for all foods
	other foods (foods with an aflatoxin risk)	afla B <sub>1</sub>	5	in force since 1990 for all foods
		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	10	in force since 1997 for all foods
	baby food	afla B <sub>1</sub>	1	in force since 2002
		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	2	
		afla M <sub>1</sub>	0.05	
	dried raisins		10	
	fruit juice	patulin	50	in force since 1997
	vegetable and fruit-berry preserves and mixes for babyfood; products for children allergic to food/lactose	afla B <sub>1</sub>	1	in force since 1980
	vegetable preserves in cans and glass jars, subproducts; beans, all seeds to be used for immediate human consumption and for processing into products for human consumption; soya press, sunflower press; all nuts; confectionery; fruit juices, fruit puree; vegetable oil		5	
	fruit-vegetable-dairy mixes for babyfood; products for children allergic to food/lactose	afla M <sub>1</sub>	0.5	
	vegetable and fruit-berry preserves and mixes for babyfood	patulin	20	in force since 1982. Tests are done if there is vegetable additive in the recipe.

(Continued)

TABLE 3.1—(Cont'd)

Country	Commodity	(Sum of) Mycotoxin(s)	Limit (µg/kg)	Remarks
	vegetables, including potatoes, fruit and grapes, berries; vegetable, fruit, berry preserves in cans and jars		50	in force since 1982
	fruit-vegetable-dairy mixes for babyfood	DON	200	in force since 1984
	all seeds to be used for immediate human consumption and for processing into the products for human consumption		1000	
	all seeds to be used for immediate human consumption and for processing into products for human consumption	T-2 toxin	100	
	beans; sunflower press;all nuts; all seeds to be used for immediate human consumption and for processing into the products for human consumption; vegetable oil	zearalenone	1000	
UNITED STATES OF AMERICA [US] 2003				
	all foods except milk	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	20	in force since 1969
	apple juice, apple juice concentrate, and apple juice component of a food that contains apple juice as an ingredient	patulin	50	in force since 2001
URUGUAY [UY] 2003 [MERCOSUR member state]				
	regulations of Uruguay in addition to MERCOSUR harmonized regulations:			
	all food and spices	afla B <sub>1</sub>	5	in force since 1994
		afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	20	
	infant food [an exception of all food and spices]		3	
	dried fruits [an exception of all food and spices]		30	
	soya protein [an exception of all food and spices]		30	
	beans	ochratoxin A	50	
	fruit juice	patulin	50	

VIETNAM [VN] 2003 foodstuffs	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>	10	in force since 1998
	total of other mycotoxins	35	
YEMEN [YE] 2003: no official regulations, but some control takes place foodstuffs	afla B <sub>1</sub> B <sub>2</sub> G <sub>1</sub> G <sub>2</sub>		
YUGOSLAVIA [SERBIA and MONTENEGRO] [YU] 2003 bean, peas spices all foodstuffs apple juice	afla B <sub>1</sub>	5	in force since 1992
		30	
	ochratoxin A	10	in force since 1990
	patulin	50	in force since 1992
ZIMBABWE [ZW] 2003: situation 1996 no regulations [FAO 1997] foods groundnuts	afla B <sub>1</sub>	5	
	afla B <sub>1</sub>	5	
	afla G <sub>1</sub>	4	

“ergot means the sclerotium or dormant winter form of the fungus *Claviceps purpurea*. The limit refers to the weight of ergot kernels per total commodity weight, and not toxin concentration.

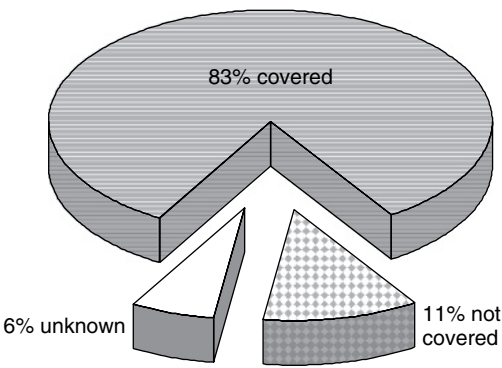


FIGURE 3.1 Percentages of world inhabitants covered and not covered by mycotoxin regulations for fruits and vegetables.

for this category of food. They cover about 83 percent of the world inhabitants (Fig. 3.1). The authors realize that, at the time of finishing this chapter (2007) the situation of 2003 may not be up-to-date anymore. Nevertheless Table 3.1 gives a global impression of the many different mycotoxin regulations that exist for this important category of food. Table 3.1 allowed a further classification into the groups (1): nuts, (2): seeds, (3): herbs and spices, (4): fruits and vegetables, (5): baby/infant food & dietary products and (6): other commodities (Fig. 3.2), and this has led to a further graphical breakdown for the first four groups in the Figs 3.2–3.5.

From Fig. 3.3 it becomes clear that the regulations for nuts almost exclusively concern aflatoxins (either B<sub>1</sub> or total aflatoxins). Both groundnuts and tree nuts are major sources of aflatoxins and therefore this situation is not surprising. Sporadically limits have been established for some other aflatoxins and the only other mycotoxin regulated in nuts is zearalenone, for which a specific limit exists in Ukraine. The rationale for this approach to regulate zearalenone in nuts at a concentration of 1000 µg/kg remains unknown.

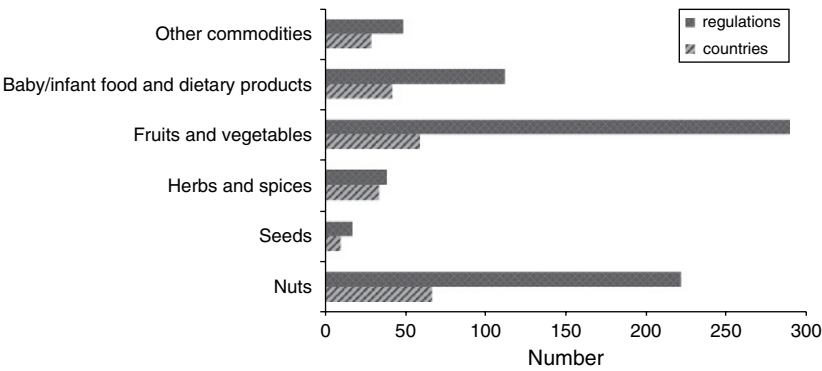


FIGURE 3.2 Number of regulating countries and number of regulations worldwide for mycotoxins in specific groups of food (products), related to fruits and vegetables.

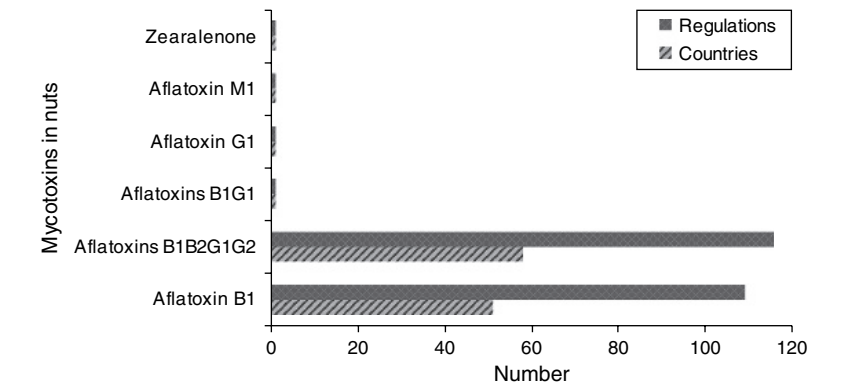


FIGURE 3.3 Number of regulating countries and number of regulations worldwide for certain mycotoxins in nuts.

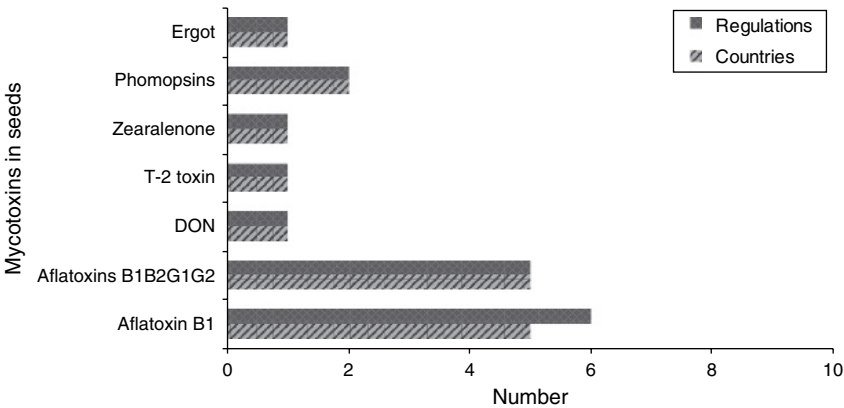


FIGURE 3.4 Number of regulating countries and number of regulations worldwide for certain mycotoxins in seeds.

Figure 3.4 shows that for seeds the number of regulating countries coincides almost with the number of mycotoxin regulations. Although the total number of worldwide mycotoxin regulations for seeds is relatively small, they encompass a variety of toxins, in contrast to the regulatory situation for nuts. Of special interest are the phomopsins, regulated in Australia/New Zealand in lupin seeds for quite some time now.

Figure 3.5 depicts the regulatory situation for mycotoxins in herbs and spices. The figure makes clear that the regulatory concern in herbs and spices is on the aflatoxins. The pattern is dominated by the countries of the European Union, where specific limits exist for both aflatoxin B<sub>1</sub> and total aflatoxins in certain specified spices. Some non-EU countries have specific limits either for aflatoxin B<sub>1</sub> or for total aflatoxins, in a few cases for both. Switzerland is the only country that has a limit for ochratoxin A in spices.

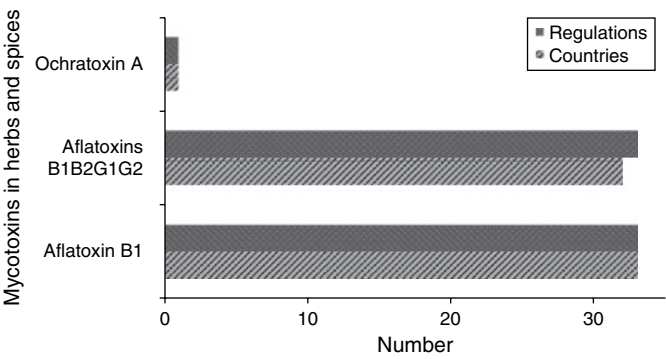


FIGURE 3.5 Number of regulating countries and number of regulations worldwide for certain mycotoxins in herbs and spices.

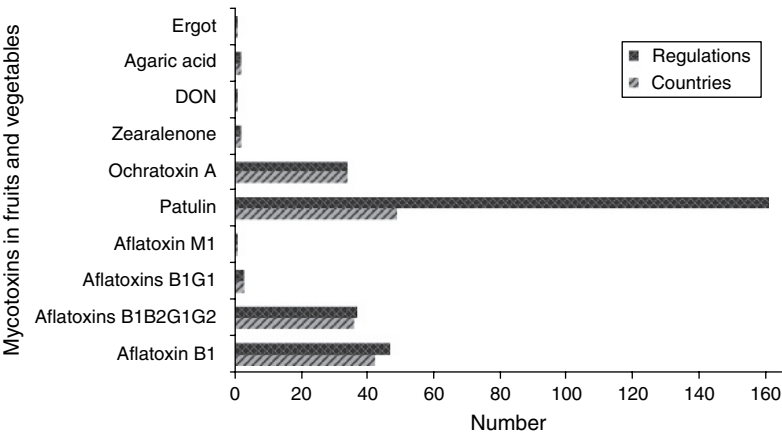


FIGURE 3.6 Number of regulating countries and number of regulations worldwide for certain mycotoxins in fruits and vegetables.

From Fig. 3.6, where the regulations for ‘fruits and vegetables *sensu stricto*’ are summarized, it is obvious that patulin is the most regulated mycotoxin. Approximately 160 different regulations exist for patulin, in some 50 countries. Patulin is not only regulated in apple (products) but as well in berries, tomato paste, mushrooms, (canned) vegetables, fermented drinks, baby food, jams, confitures and syrups. Regulations for aflatoxins and ochratoxin A exist also rather frequently, whereas sporadically specific regulations have been set for a few other mycotoxins. Of these, the regulation for aflatoxin M<sub>1</sub> may seem rather peculiar, but a closer look at this Ukrainian limit shows that the matrix concerned is a fruit–vegetable–dairy mix. The dairy component of this mix might contain aflatoxin M<sub>1</sub> and this would be an explanation for the rationale behind this regulation. An exotic mycotoxin regulated in Australia/New Zealand is agaric acid in food containing mushrooms, for which a relatively high limit has been set.

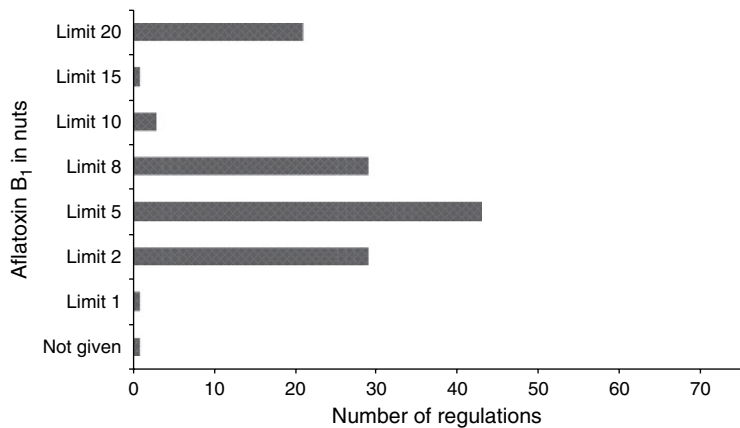


FIGURE 3.7 Frequency of limits (in µg/kg), applied in worldwide regulations for aflatoxin B<sub>1</sub> in nuts.

For the three most important toxin/matrix combinations, i.e., aflatoxins in nuts (221 regulations), ochratoxin A in fruits and vegetables (34 regulations) and patulin in fruits and vegetables (161 regulations), overviews of the regulatory limits applied are presented in Figs 3.7–3.10.

For aflatoxin B<sub>1</sub> in nuts the limits range from 1 to 20 µg/kg (Fig. 3.7) and for total aflatoxins in nuts the range is somewhat higher: 3–30 µg/kg (Fig. 3.8). This more or less reflects the occurrence ratio between aflatoxin B<sub>1</sub> and the sum of the aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub>. Many countries (e.g., the EU countries) have limits set both for aflatoxin B<sub>1</sub> and for total aflatoxins, and generally the regulatory limits are very low, given the potent carcinogenicity of the aflatoxins. Whether a regulatory level for the sum of the aflatoxins, which requires more analytical work than for aflatoxin B<sub>1</sub> alone, contributes significantly to better protection of public health than a regulatory level for aflatoxin

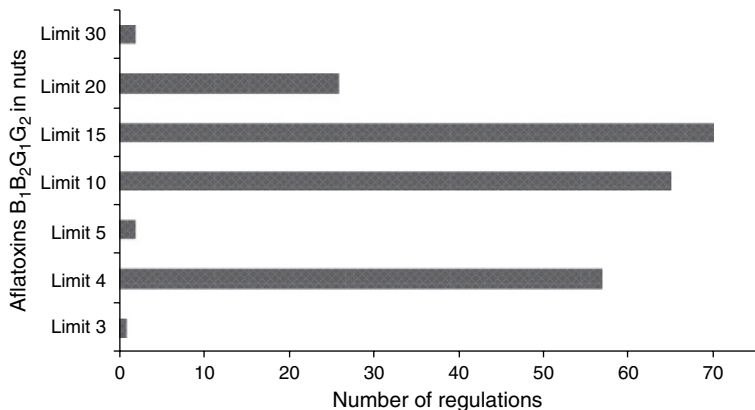


FIGURE 3.8 Frequency of limits (in µg/kg), applied in worldwide regulations for the sum of aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> in nuts.



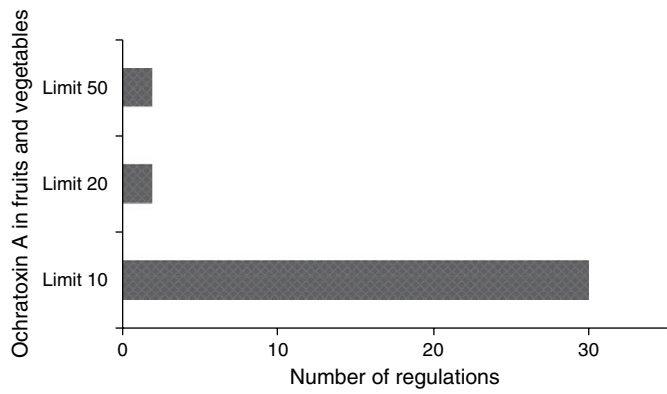


FIGURE 3.9 Frequency of limits (in µg/kg), applied in worldwide regulations for ochratoxin A in fruits and vegetables.

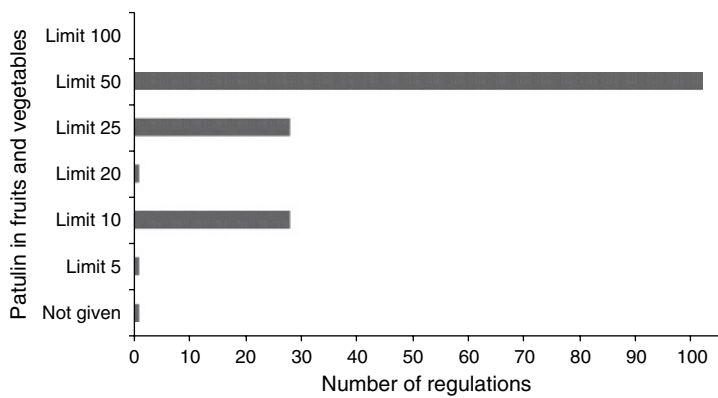


FIGURE 3.10 Frequency of limits (in µg/kg), applied in worldwide regulations for patulin in fruits and vegetables.

B<sub>1</sub> alone is debatable. Aflatoxin B<sub>1</sub> is the most important of the aflatoxins, considered from the viewpoints of both toxicology (carcinogenicity) and occurrence. It is most unlikely that commodities will contain aflatoxins B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> and not aflatoxin B<sub>1</sub>, and the concentration of the sum of the aflatoxins B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> is generally less than the concentration of aflatoxin B<sub>1</sub> alone (Food and Agriculture Organization, 2004).

For ochratoxin A in fruits and vegetables, the limits range from 10 to 50 µg/kg (Fig. 3.9) with 10 µg/kg as the dominant peak. Ochratoxin A can be found in e.g., wine and vine fruits, albeit generally at rather low levels. The significance of ochratoxin A contamination at levels around the applied legal limits remains difficult to estimate. Ochratoxin A was subject of a recent workshop, conducted by ILSI Europe in 2005 (Hazel and Walker, 2005), about ‘Ochratoxin A in food: recent developments and significance’. The conclusions were that attempts to characterize the risk associated with

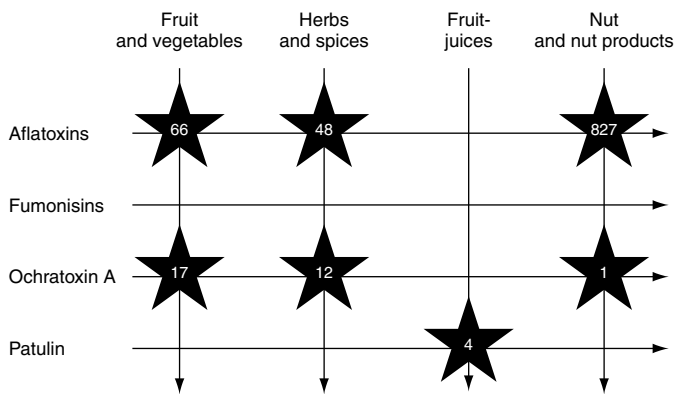


FIGURE 3.11 Number of alert notifications for different mycotoxins in vegetable diets and fruits as reported by the EU Rapid Alert System for Food and Feed in 2005.

ochratoxin A exposure were hindered because of significant gaps in current knowledge, necessary to make this risk assessment possible.

In Fig. 3.10 the range of limits that exist for patulin is shown. In contrast to the aflatoxins and ochratoxin A, no carcinogenic properties have been attributed to patulin, but this pre-eminent ‘fruit-mycotoxin’ is regulated by many countries. Limits range from 5 to 50 µg/kg. The vast majority of the 50 countries with regulations or guideline levels for patulin in fruits and vegetables apply a limit of 50 µg/kg. Patulin is one of the most regulated mycotoxins, also in international commerce. Within the Codex Alimentarius Commission, which aims to facilitate the world trade and protect the health of consumers through the development of international standards for foods and feeds, the Codex Committee on Food Additives and Contaminants (CCFAC) has established a standard for patulin in apple juice in 2003. CCFAC has also developed a code of practice for the prevention and reduction of patulin in apple juice and apple juice ingredients in other beverages (Codex Alimentarius, 2003). In practice, patulin seems to cause much less concern than aflatoxins and ochratoxin A, at least in the EU. Figure 3.11 represents a summary of the notifications done in the EU Rapid Alert System for Food and Feed (Commission of the European Communities, 2006b) in 2005 for the aflatoxins, ochratoxin A and patulin in the products relevant for this chapter. For fumonisins, no notifications were done for these products. Figure 3.11 demonstrates that aflatoxins cause most of the problems.

3.4. CONCLUSIONS

In 2004 there were approximately 100 countries in the world, which had specific regulations for mycotoxins in food and feed. The number of countries regulating mycotoxins has significantly increased over the years. Comparing

the situations in 1995 and 2004, it appears that in 2003 more mycotoxins are regulated in more commodities and products, whereas tolerance limits generally remain the same or tend to decrease. This reflects the general concerns that governments have regarding the potential adverse effects that mycotoxins can have on the health of humans and animals. Regulations have become more diverse and detailed with newer requirements regarding official procedures for sampling and analytical methodology.

Many of the current mycotoxin regulations apply directly or indirectly for fruits and vegetables. For sub-categories as nuts, seeds, herbs and spices, the regulations mainly concern the aflatoxins. For fruits and vegetables *senso stricto*, patulin is the most regulated mycotoxin. Approximately 160 different regulations exist for patulin, in some 50 countries. In addition a Codex Alimentarius standard for patulin in apple juice and a code of practice for the prevention and reduction of patulin in apple juice and apple juice ingredients in other beverages exist. This makes patulin a mycotoxin of major international regulatory concern, despite the fact that this toxin seems to cause much less problems in practice than the carcinogenic aflatoxins and ochratoxins.

## ACKNOWLEDGEMENT

The authors wish to thank the Food and Agriculture Organization of the United Nations for permission to make use of material published in 2004 in FAO Food and Nutrition Paper 81: 'Worldwide regulations for mycotoxins in food and feed in 2003'.

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# Factors Affecting Mycotoxin Production in Fruits

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## ABSTRACT

Fungi belonging to the genera *Aspergillus*, *Penicillium* and *Alternaria* are major contributors to fruit spoilage and are responsible for significant financial losses for any segment of the food industry that harvests, stores, processes or uses fruits or fruit-derived ingredients. In addition to their ability to cause fruit spoilage, these fungi produce mycotoxins that are of concern to human health. The mycotoxins most commonly associated with fruit and fruit products are patulin, aflatoxin, *Alternaria* toxins and ochratoxin A. Factors known to affect production of these mycotoxins in fruit include the fruit type and cultivar, geographical location where the fruit is grown and harvested, climate, pre-harvest treatments, method of harvest, presence of surface defects on the fruit, post-harvest treatments and storage conditions. Mycotoxin accumulation in fruits can occur in the field, during harvest, post-harvest and during storage. Gentle and sanitary handling of the fruit during harvest and in storage and processing facilities is essential for reducing fungal decay and mycotoxin production in fruits. Although consumers will likely reject fresh fruit that is visibly moldy or rotten, processed fruit products may contribute significant amounts of these toxins if decayed or moldy fruit is not removed before processing or packaging. Thus, only sound, intact fruit should be used for processed fruit products. Fruits used for dried fruit production should be dried rapidly and completely, and stored under conditions that prevent rewetting. Research is needed to identify fruit cultivars that are resistant to fungal decay and to evaluate treatments that could be used to control fungal pathogens in the field or post-harvest. Finally, more work is needed to determine the fate of mycotoxins during processing.

## 4.1. INTRODUCTION

Mycotoxins are a chemically diverse group of toxic secondary metabolites produced by filamentous fungi belonging to the genera *Aspergillus*, *Penicillium*, *Alternaria* and *Fusarium*. Of these genera, the first three are the major

contributors of mycotoxin production in fruits. These fungi are major contributors to fruit spoilage and are responsible for significant financial losses for any segment of the food industry that harvests, stores, processes or uses fruits or fruit-derived ingredients. In addition to the economic implications of their presence, *Penicillium* spp., *Aspergillus* spp. and *Alternaria* spp. produce mycotoxins that are of concern to human health. The most common mycotoxins associated with fruits are patulin, aflatoxins, *Alternaria* toxins and ochratoxin A.

Despite efforts to control fungal contamination of foods, mycotoxin-producing fungi are ubiquitous contaminants of nature and make their way into fruit in the field or orchard and at any time during harvesting, processing, storage and marketing. Although most foods have chemical and/or physical properties that permit fungal or microbial spoilage, fruits are susceptible to fungal rather than microbial spoilage due to their high water activity ( $a_w$ ) and sugar content, and presence of organic acids which impart the flesh of fruit a low pH (Tournas and Katsoudas, 2005). Defense mechanisms in fruit are fairly effective against a variety of fungi; therefore, relatively few genera and species are able to invade fruits. Some fungi are highly specialized pathogens, attacking only certain types of fruits while others have a more general ability to invade fruit tissue (Drusch and Ragab, 2003). The presence of toxin-producing fungi on fruit does not necessarily imply that mycotoxins will be present since toxin production is influenced by many factors including environmental conditions, type, cultivar and nutritional status of the fruit, the microbial load on the fruit, and strain of the fungus (Drusch and Ragab, 2003; Sanchis and Magan, 2004). It also should be noted that conditions that promote fungal growth do not necessarily coincide with those responsible for mycotoxin production. Mycotoxins can be present in intact (fresh) fruits, juices, wines, canned fruits and dried fruit products.

Efforts have been made to understand the circumstances by which mycotoxigenic fungi infect fruit and produce toxins so that conditions can be made unfavorable for toxin production. This chapter will review the factors resulting in accumulation of patulin, aflatoxins, ochratoxin and *Alternaria* toxins in fruit products.

## 4.2. PATULIN

### 4.2.1. GENERAL ASPECTS

Patulin is a low molecular weight  $\alpha,\beta$ -unsaturated  $\gamma$ -lactone that is produced by fungi belonging to several genera including *Penicillium*, *Aspergillus* and *Byssoschlamys*. Although patulin can occur in many moldy fruits including pears, berries, mangoes, plums, apricots and tomatoes, the major source of patulin contamination is apples with blue rot, and in apple cider or juice pressed from moldy fruit. *Penicillium expansum* is believed to be the major fungal species contributing to patulin in apple products. Patulin has been demonstrated to be acutely toxic (Dailey et al., 1977), genotoxic (Alves

et al., 2000), teratogenic (Dailey et al., 1977; Roll et al., 1990; Sugiyanto et al., 1993) and possibly immunotoxic (Sorenson et al., 1985; Escuola et al., 1988) to animals. At present, there are no published toxicological or epidemiological data to indicate whether consumption of patulin is harmful to humans. However, there is a desire to limit patulin levels in apple products since infants and young children are major consumers of these foods and the effects of long-term exposure to patulin are not yet known. Many countries, including the United States, have set regulatory limits for patulin in apple products of 50 µg/L or less (van Egmond, 1989; U.S. Food and Drug Administration, 2001; Drusch and Ragab, 2003).

#### 4.2.2. OCCURRENCE OF PATULIN IN FRUIT

Patulin is found with greater incidence and concentration in apples than in other fruit, and they contribute to the vast majority of patulin in the human diet (FDA, 2001). The toxin has been detected in intact fruit, juice, cider, apple sauce and apple puree. Whole apples (table fruit) are not believed to contribute significantly to human exposure since contaminated fruit is often discarded or trimmed to remove moldy areas before it is eaten (Jackson and Dombrink-Kurtzman, 2006). The greatest exposure to patulin comes from consumption of apple juice and cider pressed from moldy fruit (FDA, 2001).

Numerous surveys have been published on the incidence and concentration of patulin in apples and apple products (Scott et al., 1972; Wilson and Nuovo, 1973; Lindroth and Niskanen, 1978; Mortimer et al., 1985; Watkins et al., 1990; Burda, 1992; De Sylos and Rodriguez-Amaya, 1999; Yurdun et al., 2001; Leggott and Shephard, 2001; Roach et al., 2002; Ritieni, 2003). Harwig et al. (1973) surveyed 61 samples of whole apples from different orchards in Canada. *P. expansum* was isolated from 42 of the samples, while patulin was found in 28 samples of expressed juice at levels up to 240 µg/L. Wilson and Nuovo (1973) analyzed 100 samples of freshly pressed apple cider and detected high levels of patulin (up to 45 000 µg/L) in several samples of cider produced from organically grown fruit. The authors concluded that cider samples with the highest patulin concentrations were made from ground-harvested and rotten fruit. While Malmauret et al. (2002) and Ritieni (2003) reported no significant difference in patulin levels from fruit grown organically compared to conventionally grown fruit, Piemontese et al. (2005) found the opposite to be true. Overall, surveys of apple products indicate that although the incidence of patulin contamination is fairly high, levels of contamination are typically less than 50 µg patulin/L. Surveys of the presence of patulin in cherries, strawberries, raspberries, mulberries, peaches, grapes, pears and other fruits indicated low incidence and levels of the toxin (Frank, 1977; Drusch and Ragab, 2003; Piemontese et al., 2005).



### 4.2.3. FACTORS AFFECTING FORMATION OF PATULIN IN APPLES

#### 4.2.3.1. Introduction

A variety of fungi are reported to be capable of producing patulin in defined media including *Aspergillus clavatus*, *A. giganteus*, *A. terreus*, *Byssosclamyces fulva*, *B. nivea*, *Paecilomyces variotii*, *Penicillium carneum*, *P. clavigerum*, *P. concentricum*, *P. coprobium*, *P. dipodomyicola*, *P. expansum*, *P. glandicola*, *P. roqueforti*, *P. sclerotigenum* and *P. vulpinum* (Scott, 1974; Bullerman, 1979; Palmgren and Ciegler, 1983; Hasan, 2000). However, *P. expansum* is considered the major producer of patulin in fruit.

The optimum growth temperature for *P. expansum* is near 25°C, but there are reports of growth of the organism at -3°C (Pitt, 2002). Minimum water activities for spore germination are 0.82–0.83 (Northolt et al., 1978). *P. expansum* has a very low requirement for oxygen; the organism was found to grow at atmospheric oxygen levels of less than 2 percent. Carbon dioxide concentrations of up to 15 percent have been found to stimulate growth of the organism (Pitt, 2002). The growth characteristics of the fungus help explain the finding of *P. expansum* and patulin in apples stored under modified atmosphere conditions (Lovett et al., 1975b).

The optimum temperature for patulin production has been reported in the range of 23–25°C (Paster et al., 1995; McCallum et al., 2002). Although patulin production tends to decrease as temperature is decreased, patulin can be produced at low temperatures (0–4°C). Consequently, refrigerated storage is not practical to inhibit patulin production in fruit (Sommer et al., 1974a; Hasan, 2000).

At present, there are a number of factors known to affect production of patulin in apple products including apple cultivar, geographical location where the fruit is grown and harvested, climate, pre-harvest treatments, method of harvest, surface defects on the fruit, post-harvest treatments and storage conditions. At present, it is not clear which of these factors plays the greatest role in mycotoxin production or how they can be manipulated to prevent or reduce patulin contamination of apple products. A better understanding of the aspects influencing patulin production may aid in developing effective means for controlling patulin levels in food. Following is a description of some factors known to affect patulin production in apples.

#### 4.2.3.2. Physical, chemical and microbial properties of apples

Several investigators have shown that fruit cultivars differ in their susceptibilities to *P. expansum* rot and to patulin formation in apple tissue (Wilson and Nuovo, 1973; Beer and Amand, 1974; Northolt et al., 1978; Paster et al., 1995; Spotts and Mielke, 1999; Martins et al., 2002; McCallum et al., 2002; Jackson et al., 2003). The differences in cultivars may be due to unique physical and chemical characteristics of each cultivar such as skin thickness and strength, flesh firmness, pH of flesh, sugar levels, levels of antimicrobial compounds and other apple constituents (Beer and Amand, 1974; Damoglou et al., 1985;

McCallum et al., 2002). Apple cultivars with an open calyx are at greater risk for patulin development within the apple core (Codex Alimentarius Commission, 2002). Since core rot is often not detected, juice or cider pressed from affected fruits may have high patulin levels.

Microbiological factors can influence patulin formation. The microbiological flora on apples differ according to geographic area, climatic conditions, pesticide or fungicide treatments, cultivar, presence of competitive microorganisms, harvest method and post-harvest treatments (Doores, 1983). Spores of *P. expansum* are found in soil, on plant surfaces and in air and are transferred to dump tank and flume water in packinghouses by contaminated wooden picking bins and fruit (Sanderson and Spotts, 1995). A report by Sommer et al. (1974b) indicates that the presence of a patulin-producing species does not necessarily imply patulin production in apples. Factors like incubation temperature, lesion size and substrate also play important roles (Drusch and Ragab, 2003). Substantial differences have been noted among *P. expansum* strains in terms of growth kinetics and patulin production (McCallum et al., 2002). McCallum et al. (2002) found that the highest patulin levels were those from isolated strains displaying aggressive growth and profuse mycelial development.

#### 4.2.3.3. Pre-harvest factors

Although patulin production in fruit is believed to occur mainly post-harvest, several factors pertaining to the growing conditions of fruit trees may influence fungal infection and mycotoxin production in apples. Codex Alimentarius Commission (2002) outlined Good Agricultural Practices (GAP) that may reduce the likelihood of infection of fruit trees. Trees should be trimmed of dead and diseased wood and mummified fruits and pruned to allow proper air flow and light penetration (Codex Alimentarius Commission, 2002). It has been demonstrated that supplementing fruit trees with foliar calcium sprays during the growing season and use of minimal amounts of nitrogen reduce pre-harvest infection of apple fruit by fungi (Conway, 1982; Agriculture and Agri-Food Canada, 2003). When ammonium molybdate was applied as a pre-harvest treatment to apple trees, a significant reduction in blue mold decay was observed in the treated apples after three months of cold storage (Nunes et al., 2001).

Post-harvest decay can be reduced by pre-harvest applications of fungicides. Studies on the effectiveness of applications of ziram fungicide showed an average reduction in decay of 25–50 percent (Sugar and Spotts, 1995; Sholberg and Conway, 2001). Synthetic fungicides are being developed to protect produce from a number of post-harvest diseases. During the past decade, biological control of post-harvest fungal diseases with naturally occurring antagonists (yeasts, yeast-like fungi and bacteria) has become an alternative to synthetic fungicide control (Nunes et al., 2002). Few strains of these antagonists, however, have been developed as biofungicides for commercial application because of high cost of development and commercialization and because of inconsistent activity (Lima et al., 2005). More work is needed to determine the efficacy of

pre-harvest biocontrol of *P. expansum* and to determine if biocontrol affects post-harvest formation of patulin in fruit.

#### 4.2.3.4. Harvest factors

The condition of fruit (degree of ripening, microbial load, presence of skin defects, etc.) at harvest determines the length of time that it can be stored (Sholberg and Conway, 2001). Stage of maturity at harvest is believed to be one of the main factors determining the susceptibility of fruit to mechanical damage and to blue mold rot during post-harvest storage. Fruits become increasingly susceptible to fungal invasion during ripening as the pH of the tissue increases, soluble sugars build up, skin layers soften and defense barriers weaken (Aziz and Moussa, 2002; Torres et al., 2003). To reduce undesirable biochemical changes, apples should be picked when mature but not fully ripe to ensure that they can be stored for several months (Sholberg and Conway, 2001). Studies indicate that bruising and skin punctures substantially increase the susceptibility of fruit to decay. Gentle handling of fruit by pickers during harvest and care during transport of the fruit from the orchard to the packinghouse, juice processing plant or storage may prevent injury to the fruit (Janisiewicz, 1999).

Since rain during harvest allows for increased fungal contamination and infection, fruit should be harvested in dry weather conditions and quickly transferred to cold storage. Fallen fruit in the orchard should be discarded and not sold in the fresh market or used in processed apple products. Jackson et al. (2003) reported significantly higher patulin levels in cider produced from ground-harvested apples than from tree-picked fruit. Fallen apples may have cuts or cracks in the peel that become infected from fungal spores from the soil.

One of the major methods for controlling *P. expansum* infection of apples is improved sanitary practices during harvest (Spotts and Cervantes, 1994; Agriculture and Agri-Food Canada, 2003). This includes reducing contamination of packing/storage bins with orchard soil by cleaning and sanitizing bins before use. Studies by Spotts and Cervantes (1994) found that while steam was the most effective treatment on wood and plastic bins, chlorine compounds, sodium *o*-phenylphenate (SOPP) and quaternary ammonia compounds were also effective sanitizing agents.

#### 4.2.3.5. Post-harvest factors

Since the majority of patulin is formed in fruit post-harvest, considerable efforts have been devoted to developing strategies for reducing proliferation of fungal pathogens and contamination with patulin during storage. Organic matter (soil, plant material, decayed apples) can act as reservoir of fungal spores that can contaminate fruit. Therefore, maintaining sanitary conditions in all areas where fruit is packaged, stored and processed is essential for preventing fungal contamination. Water systems (water flumes), used to float apples from field bins to bulk tanks, minimize mechanical damage to fruit. Flume water typically contains chlorine (sodium hypochlorite) or

sodium-*o*-phenylphenate (SOPP) to reduce fungal spore load (Blanpied and Purnasiri, 1968; Sholberg and Conway, 2001). Active chlorine levels in flume water must be maintained periodically to ensure that spores are destroyed.

Apples are typically washed in dump tanks containing water or chlorinated water, with brusher-scrubbers and/or with high-pressure water sprayers (Root, 1996; Wright et al., 2000). Since *P. expansum* and patulin are associated with the soft rot of apples, washing may result in the removal of rotten areas of the apple and the partitioning of patulin into the cleaning water (Sydenham et al., 1995; Acar et al., 1998; Jackson et al., 2003). In a recent study, Okull and Laborde (2004) reported that electrolyzed oxidizing water has potential as an alternative to chlorine disinfectants for controlling infection of apples by *P. expansum* during handling and processing. Total removal of patulin during the wash treatments is unlikely since patulin can diffuse up to 1 cm into the healthy tissue (Taniwaki et al., 1992).

Removal of decayed or damaged fruit, or trimming moldy portions of apples prior to packaging or processing has been reported to reduce patulin levels in apple juice (Lovett et al., 1975a; Taniwaki et al., 1992; Sydenham et al., 1995; Sydenham et al., 1997; Beretta et al., 2000; Jackson et al., 2003). Although removing visibly decayed fruit before processing is a proven method for reducing patulin levels in apple products, there is no guarantee that culling alone can totally eliminate patulin. Apples with “invisible” sources of fungal rot (core rots) can contaminate apple juice, cider or puree with patulin if they are not removed before processing.

After harvest, apples are generally kept in cold storage at  $-1$  to  $3^{\circ}\text{C}$  with or without modified atmospheres. These treatments can extend the shelf life of apples from 9 to 28 weeks, depending on apple cultivar (Moodley et al., 2002). Since apple cultivars differ in their susceptibility to post-harvest diseases, cultivars with resistance to mechanical damage and infection should be chosen, especially if they will be kept in long-term storage. The growth of *P. expansum* and production of toxin has been shown to be reduced during cold storage (Beer and Amand, 1974; Barkai-Golan, 1990; Paster et al., 1995; Morales et al., 2007) suggesting that apples should be kept in refrigerated storage when possible to slow mold growth and reduce mycotoxin production.

Treatments that have shown promise at reducing patulin levels in apple juice include use of filtration, centrifugation treatments, use of charcoal, addition of ascorbic acid and fermentation (Brackett and Marth, 1979; Leggott et al., 2000, 2001; Bissessur et al., 2001; Kadakal and Nas, 2002; Moodley et al., 2002; Moake et al., 2005). Bissessur et al. (2001) evaluated the effectiveness of several clarification processes for the reduction of patulin in apple juice. Pressing followed by centrifugation resulted in 89 percent reduction in levels of the toxin. Patulin reductions using paper filtration, enzyme treatment and fining with bentonite were 70, 73 and 77 percent, respectively. These data suggest that patulin tends to bind to the apple solids, which are removed from the juice during treatment.

### 4.3. AFLATOXIN

#### 4.3.1. GENERAL ASPECTS

Aflatoxins are a family of polyketide mycotoxins produced in agricultural products primarily by *A. flavus* and *A. parasiticus*. Although many different kinds of aflatoxins have been isolated from nature, the major aflatoxins found in agricultural commodities include aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), B<sub>2</sub> (AFB<sub>2</sub>), G<sub>1</sub> (AFG<sub>1</sub>) and G<sub>2</sub> (AFG<sub>2</sub>). *A. flavus* is a common constituent of the microflora in air and soil throughout the world and produces aflatoxins when the fungus colonizes a commodity, grows and finds conditions appropriate for toxin production. Major agricultural products that have been found to be infected with *A. flavus* or *A. parasiticus* include corn, peanuts, cottonseed, rice, tree nuts, coffee beans, cereal grains and fruits. Infection may occur in the field, particularly when the plant experiences water, temperature or nutrient stress and accumulation of the toxin may increase if the harvested crop is improperly stored. Strains of *A. flavus* vary greatly in their ability to produce aflatoxins. For example, toxigenic strains of *A. flavus* typically produce only AFB<sub>1</sub> and AFB<sub>2</sub> but most strains of *A. parasiticus* typically produce all four toxins. In addition to aflatoxins, many strains of *A. flavus* produce the mycotoxin cyclopiazonic acid, which is also a natural contaminant of a variety of crops (Lee and Hagler, 1991).

Aflatoxins are among the most potent carcinogenic, teratogenic and mutagenic compounds in nature. AFB<sub>1</sub> is listed as a group I carcinogen by the International Agency for Research on Cancer (IARC) and is implicated as being the cause of human primary hepatocellular carcinoma (IARC, 2002). The quantity of aflatoxins in food and feed is closely monitored and regulated in most countries. For example, the U.S. Food and Drug Administration (U.S. Food and Drug Administration, 2000) action level for total aflatoxins in food is 20 ng/g. In the European Union, aflatoxin levels in several spices (paprika, nutmeg, etc.) are regulated with maximum residue levels of less than 5 ng/g for AFB<sub>1</sub> and 10 ng/g for total aflatoxin. In peanuts, nuts, dried fruit, cereals and processed food products intended for direct food consumption, the maximum level has been set at 2 ng/g for AFB<sub>1</sub> and 4 ng/g for total aflatoxins (European Commission, 2002).

#### 4.3.2. OCCURRENCE OF AFLATOXIN IN FRUIT

Many reports have shown that under experimental conditions a variety of fruits (figs, dates, citrus fruits, pineapple, apricots, cherries, blackberries, strawberries, grapes) can support the growth of *A. flavus*, resulting in aflatoxin formation (Alderman and Marth, 1974a,b,c; Morton et al., 1979; Llewellyn et al., 1982). However, aflatoxin has only been found to naturally occur in fruits such as figs, dates and citrus fruits grown in regions having the high temperature conditions necessary for growth of aflatoxigenic fungi (Emam et al., 1994; Shenasi et al., 2002a,b; Drusch and Ragab, 2003; Karaca and Nas, 2006).

Of the fruits vulnerable to aflatoxin contamination, figs are considered to be one of the most susceptible due to their chemical properties and the ability of *A. flavus* to enter and colonize the internal cavity of the fruit (Buchanan et al., 1975; Doster et al., 1996).

The few surveys that have been conducted on the aflatoxin levels in fruit products obtained in Egypt (Zohri and Abdel-Gawad, 2007), Spain (Blesa et al., 2004), Turkey (Senyuva et al., 2007), India (Sharma and Sumbali, 1999) and the United Kingdom (Sharman et al., 1991; MacDonald et al., 1999) have shown relatively low levels of the toxin. However, Özay and Alperden (1991) reported levels as high as 78 ng/g AFG<sub>1</sub> and 63 ng/g AFB<sub>1</sub> in figs. In a survey of aflatoxigenic fungi and toxin formation in fresh figs (not dried) grown in California, Doster et al. (1996) found that of 25 samples of figs contaminated with *A. flavus* or *A. parasiticus*, 13 did not contain aflatoxin, while five samples contained aflatoxin levels greater than 1000 ng/g. Senyuva et al. (2007) measured aflatoxin levels in dried figs for export from Turkey from crop years of 2003–2006 and found that less than 4.0 percent of fig samples were contaminated with greater than 2 ng/g AFB<sub>1</sub> while less than 5.1 percent of fig samples contained more than 4 ng/g total aflatoxins. A survey of dates grown in the United Arab Emirates showed levels as high as 113 and 133 ppb of AFB<sub>1</sub> and AFG<sub>1</sub>, respectively (Ahmed and Robinson, 1998). Sharma and Sumbali (1999) reported high levels (96–8164 ng/g) of AFB<sub>1</sub> in dried quince slices purchased in India. Although aflatoxins have been experimentally produced on intact citrus fruits, little information exists on the natural occurrence of these toxins in these fruits.

### 4.3.3. FACTORS AFFECTING FORMATION OF AFLATOXIN IN FRUIT

#### 4.3.3.1. Introduction

Aflatoxigenic molds are very common and can grow on numerous substrates under many conditions, but thrive in hot, humid, subtropical and tropical climates. Optimum conditions for growth of *A. flavus* and *A. parasiticus* on defined media are 35°C and 0.95 $a_w$ , while those for aflatoxin production are 33°C and 0.99 $a_w$  (Sanchis and Magan, 2004; Abbas, 2005). Minimum  $a_w$  for germination and growth of *A. flavus* and *A. parasiticus* in the temperature range of 30–52°C is 0.80–0.82 $a_w$  (Pitt and Miscamble, 1995). Alderman and Marth (1974a) evaluated the effects of RH on aflatoxin production on the surface of intact citrus fruits. Surfaces of grapefruits, lemons, limes and oranges were inoculated with *A. parasiticus* spores, then stored at various relative humidities (RH) for 14 days at 27°C. RH values of less than 66% resulted in reduced mold growth and aflatoxin formation. At RH values between 66 and 93%, mold growth was extensive and aflatoxin levels began to be detected in the fruit. However, aflatoxin levels in the fruit were not influenced by RH values (Alderman and Marth, 1974a).

Some of the chemical constituents of fruits have been shown to affect the growth of aflatoxigenic fungi and production of aflatoxins. Presence of high soluble solids has been found to be a major factor affecting aflatoxin production in in vitro experiments. Alderman and Marth (1974b,c) studied the chemical factors that affect aflatoxin production in grapefruit juice by *A. flavus* and *A. parasiticus*. As the percentage of soluble solids in the juice increased, more AFB<sub>1</sub> was produced by *A. flavus* while *A. parasiticus* produced more AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub>. Grapefruit peel was found to be a better substrate than grapefruit juice for toxin production, presumably because the peel contains more carbohydrate and nitrogenous compounds than juice (Alderman and Marth, 1974b). Mateles and Adye (1965) showed in defined media that a 1 percent concentration of sucrose, glucose and fructose supported mold growth but not aflatoxin formation, while Davis et al. (1966) showed that when the concentration of sucrose in defined media was increased from 5 to 10 percent and from 10 to 20 percent, the yield of aflatoxin also doubled.

Aflatoxigenic fungi can grow and form aflatoxin on virtually any agricultural commodity given the proper temperature, moisture and level of aeration (Alderman and Marth, 1974c). For example, aflatoxin formation was demonstrated in pineapple, cooked apricot, strawberries, blackberries, cherries, whole citrus fruits, orange juice, and grapefruit juice inoculated with *A. flavus* or *A. parasiticus* and held under controlled conditions (Alderman and Marth, 1974a,b,c; Morton et al., 1979; Llewellyn et al., 1982). However, a few reports exist on the natural occurrence of aflatoxins in these products. Most of the information on factors affecting aflatoxin formation refers to aflatoxin accumulation in figs and dates. Following is a description of some factors known to affect toxin formation in these fruits.

#### 4.3.3.2. Effects of pre-harvest, harvest and post-harvest conditions on aflatoxin formation in dates and figs

Date fruits are grown in areas such as the Arabian peninsula, where the temperatures and RH are relatively high. These climatic conditions in addition to the color, flavor and chemical changes when the fruits ripen make dates susceptible to fungal attack (Ahmed and Robinson, 1998). In the first stage of maturation (Kimri), the date fruit is green and inedible. In the second stage (Khalal), the fruit is mature, full-colored and most popular for direct (fresh) human consumption. The fruit then progresses to the Rutab stage where the fruit is soft and brown. The fruit at this stage is harvested, then dried to the final stage (Tamr) where it is immediately consumed or stored for up to 1 year (Shenasi et al., 2002a,b). Shenasi et al. (2002a,b) examined 25 varieties of dates for microbial counts and aflatoxin levels immediately after harvest and after storage for 14 days at 30°C and 98 percent RH. Microbial counts were high in fresh dates harvested in the Kimri stage of maturation, increased dramatically in the Khalal stage, then decreased significantly in the Rutab



stage. No aflatoxin was detected in any samples of fresh dates. However, after 14 days of storage, 3 out of the 25 varieties of dates (12 percent) showed detectable levels of AFB<sub>1</sub> (Shenasi et al., 2002a,b), but only when dates were harvested in the inedible Kimri stage. In contrast, Ahmed et al. (1997) found that aflatoxin formation was highest in artificially inoculated (*A. parasiticus*) dates in the Khalal stage of maturation, with maximum levels of total aflatoxins greater than 300 µg/g. Total aflatoxins were detected at levels less than 100 µg/g in dates in Kimri and Rutab stages. In general, the late-maturing varieties of dates had less aflatoxin formation than early-maturing varieties (Ahmed et al., 1997). Overall these studies suggest that dates may be contaminated with aflatoxins if the fruits are harvested in the earlier stages of maturation and if stored under conditions of high RH. Insect, bird or harvest-related damage which exposes inner tissues of the fruits to toxigenic aspergilli may also result in aflatoxin contamination (Ahmed et al., 1997). Removal of decayed or damaged dates before drying and packaging may reduce the incidence of aflatoxin contamination.

Several researchers have studied the conditions resulting in aflatoxin accumulation in figs. Fig fruits contain high levels of carbohydrates which make them an attractive substrate for aflatoxigenic fungi. In addition, the temperatures in regions where figs are grown (27–30°C) are within the range optimal for mold growth and mycotoxin formation (Anton, 1988). Buchanan et al. (1975) found that immature fig fruits are resistant to invasion by *A. flavus* but that as the fruits ripen and soften they lose their resistance. Fungal spores on the surfaces of intact ripe fig fruits are able to infect and colonize the fruits, suggesting that *A. flavus* can penetrate the fruit skin (Buchanan et al., 1975). The fungal pathogen may also enter the fruit through the ostiole or through minute wounds in the fruit skin caused by insects or mites (Doster et al., 1996).

Contamination of figs with aflatoxins can start on the tree and can continue during harvest and during storage. Figs are typically left on the tree until they are fully ripe and then shrivel and fall to the ground. The ground-harvested fruits are typically sun dried for up to 5 days to reduce the moisture content of the fruit. Karaca and Nas (2006) reported that aflatoxins can accumulate in figs when figs are harvested under conditions of high RH and sudden rains. Özay et al. (1995) found that harvesting figs by hand and drying them as soon as possible with a solar drier reduced aflatoxin levels in dried figs. Once dried to  $a_w$  less than 0.80 and stored under conditions that prevent rewetting of the dried fruits, aflatoxin is not formed (Buchanan et al., 1975; Drusch and Ragab, 2003). Steiner et al. (1988) reported that bright greenish yellow fluorescence under UV light was strongly correlated to the presence of aflatoxin in dried figs. Use of UV light to sort aflatoxin-contaminated figs from sound fruit was found to be an effective method for reducing aflatoxin levels in batches of figs on an industrial scale (Steiner et al., 1988). While this technique is effective at sorting out figs that are contaminated with aflatoxins on the surface of the fruit, it may not be effective at identifying fruit with internal contamination (Doster and Michailides, 1998; Drusch and Ragab, 2003).



## 4.4. ALTERNARIA TOXINS

### 4.4.1. GENERAL ASPECTS

*Alternaria* is a common fungal genus with a number of species that cause pre- and post-harvest damage to agricultural products including cereal grains, fruits and vegetables. Under suitable conditions, some of these species produce a range of mycotoxins and other metabolites. *Alternaria alternata* is the most important mycotoxin-producing species and has been found to grow on cereals, sunflower seeds, olives and various fruits (Scott, 2001; Scott, 2004).

Strains of *A. alternata* produce a chemically diverse group of mycotoxins including alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TEA), altenuene (ALT) and altertoxins (AT) (Scott, 2004). *Alternaria* toxins have been implicated in animal and human health disorders. Toxicity tests with the individual mycotoxins indicate that AOH and AME are not very acutely toxic, but are mutagenic (Brugger et al., 2006; Schrader et al., 2006). AT and related perylene derivatives are acutely toxic and potent mutagens (Stack and Prival, 1986; Schrader et al., 2001; Scott, 2004). TEA is not mutagenic in bacterial assays, but is toxic to several animal species such as dogs and chickens (Scott, 2004). TEA has indirectly been associated with the human hematological disorder known as Onyala (Logrieco et al., 2003). At present, there are no regulatory limits for *Alternaria* toxins in foods.

### 4.4.2. OCCURRENCE OF ALTERNARIA TOXINS IN FRUITS

Molds of the genus *Alternaria* are widely distributed in the environment. They are present in soil and many species are pathogens that cause diseases in cereal plants, vegetable and fruit crops in the field, or decay in crops after harvest and during storage. Since these fungi grow well at low temperatures, they are responsible for spoilage of fruits and vegetables in refrigerated storage (Stinson et al., 1980). The production of mycotoxins by *Alternaria* spp. can occur in the field and during harvest, transportation and storage.

*Alternaria* toxins (AOH, AME, TEA and in some cases AT and ALT) have been detected in fruits (tomatoes, tangerines, cucumbers, mandarin oranges, lemons, melons, peppers, apples and olives) having visible *Alternaria* rot (Stinson et al., 1981; Hasan, 1995; Scott, 2001). Levels of AOH and TEA in apples were reported to be less than 58 800 and 500 ng/g, respectively (Scott, 2001). Tomatoes contained up to 5300 ng/g AOH and 139 000 ng/g TEA (Scott, 2001). Levels of AOH and TEA in mandarin oranges were less than or equal to 5200 and 173 900 ng/g, respectively (Scott, 2001). Peppers contained up to 440 000 ng/g AOH, 294 000 ng/g AME, 103 000 ng/g ALT and 342 000 ng/g TEA (Scott, 2001). Olives were found to contain up to 2300, 2900 and 1400 ng/g AOH, AME and ALT, respectively (Scott, 2001). In inoculation experiments, Tournas and Stack (2001) and Stinson et al. (1980, 1981) were able to demonstrate

production of AOH, TEA, AME and ALT in apples, tomatoes, berries, grapes, oranges and lemons. These studies indicate that these fruits have the potential for toxin contamination, given the proper conditions for fungal growth.

Since consumers will reject fruit that is visibly moldy or rotten, whole fresh fruits are not believed to contribute significant amounts of *Alternaria* toxins to human exposure. However, processed fruit products may contribute significant amounts of these toxins to the human diet if decayed or moldy fruit is not removed before packaging or processing. Limited information exists on the levels of *Alternaria* toxins in processed fruit products. TEA has been detected in canned tomato juice, pulp, puree, paste and whole stewed tomatoes at levels up to 178 ng/g (da Mota and Valente-Soares, 2001). Lau et al. (2003) and Delgado and Gomez-Cordoves (1998) confirmed the presence of AOH in apple juice, grape juice, cranberry nectar, raspberry juice, red wine and prune nectar at levels up to 6 ng/g. Although several investigators demonstrated production of toxins in *A. alternata*-inoculated citrus fruits and grapes, there is a lack of information on the natural occurrence of *Alternaria* toxins in these fruits and products derived from these fruits. More information is needed on the natural occurrence of *Alternaria* toxins in processed fruit products.

### 4.4.3. FACTORS AFFECTING FORMATION OF *ALTERNARIA* TOXINS IN FRUIT

#### 4.4.3.1. Introduction

The genus *Alternaria* is widely distributed in soil and on plant surfaces, with many species known to be plant pathogens. Species are known to grow at low temperatures and have been predominantly associated with spoilage of fruit and vegetables during refrigerated transport and storage. *A. alternata* is a common species found in areas under arable crop production. *Alternaria* spp. are environmentally flexible pathogens and can thrive in areas of high rainfall as well as arid conditions. Few studies have determined the optimal conditions responsible for germination, growth and mycotoxins production in *Alternaria* spp. Sanchis and Magan (2004) have reported that the  $a_w$  needed for *A. alternata* to germinate, grow and produce mycotoxins (AE, AME and AOH) are greater than or equal to  $0.86a_w$ . Optimum production of the toxins was at  $a_w$  greater than 0.97 (Sanchis and Magan, 2004). Hasan (1996) found that the optimum temperature for toxin production in synthetic media by *A. alternata* IMI 89344 was 28°C for AOH and AME, 21°C for TEA and 14°C for AT.

#### 4.4.3.2. Pre-harvest, harvest and post-harvest conditions

**Apples.** *Alternaria alternata* is responsible for moldy-core rot in some apple cultivars (Red Delicious). The fungus colonizes the flower parts and eventually grows, possibly through the open calyx tube, to the core or carpel regions during fruit development and storage. Moldy-core is characterized by the

growth of the mycelia within the locules, with or without penetration into the mesoderm (Reuveni et al., 2002). Once internalized in the fruit, the fungus can proliferate and produce toxins.

Conditions that affect infection in the orchard include high relative humidity, mild temperatures (10–25°C) and tissue susceptibility (Reuveni et al., 2002). Artificial inoculations revealed that the beginning of bloom and full bloom were the most susceptible developmental stages for infection with *A. alternata* (Reuveni et al., 2002). Treating trees with foliar applications of fungicides,  $\beta$ -aminobutyric acid, or potassium phosphate starting at the beginning of bloom until fruit set reduced the number of infected fruits by over 40 percent (Reuveni et al., 2002; Reuveni et al., 2003).

Since *A. alternata* rot occurs in the apple core, it is unlikely that cleaning treatments are effective at reducing fungus or mycotoxins levels. Apples with moldy core may be used in the production of apple juice and other processed apple products since the defects are hidden and not detected upon inspection. Use of contaminated apples may result in high levels of *Alternaria* toxins in processed apple products. AOH and AME were shown to be stable in apple juice with and without added vitamin C over 20 days at ambient temperatures, and when heated at 80°C for 20 min (Scott, 2001). Methods are needed to detect the presence of moldy cores in batches of apples so that they can be removed before pressing and processing.

**Citrus fruits.** *Alternaria* brown spot is a disease in tangerines and related citrus fruits caused by *A. alternata*. The pathogen attacks immature leaves, twigs and fruit, causing necrotic lesions that may appear as early as 24 hours after infection (Magnani et al., 2007). The fungus also produces lesions on more mature fruit, ranging from small, necrotic spots to large, sunken pockmarks. The pathogen causes considerable crop losses and blemishes on fruit that are unacceptable to consumers (Timmer et al., 2000). Although the relationship between the presence of lesions and mycotoxins in citrus products is not currently known, fruit with these defects should not be used to produce juice.

Release of conidia of *Alternaria* spp. on citrus is triggered by rainfall events and by sudden changes in relative humidity (Timmer et al., 1998). Cultural measures that have been shown to reduce susceptibility of citrus trees to *Alternaria* brown spot disease include wider tree spacing, elimination of overhead irrigation and avoidance of high nitrogen fertilizers and excessive watering (Canihos et al., 1999; Timmer et al., 2000; Akimitsu et al., 2003). Fungicide applications have been shown to be essential for disease control (Timmer et al., 2000). Research is needed to determine the effects of these control methods on the incidence and levels of *Alternaria* toxins in citrus products.

**Tomatoes.** Tomatoes and other soft-skinned fruits are particularly susceptible to invasion with *A. alternata* (Hasan, 1995; Hasan, 1996; da Mota and Valente-Soares, 2001; Andersen and Frisvad, 2004). In tomatoes, *A. alternata* requires injured or weakened tissue for penetration and growth. In the presence of water on the ripening fruit from rain, dew or overhead

irrigation, fungal spores can germinate on the surface of the fruit. The fungus can penetrate the tomato skin through growth cracks, insect injuries, skin punctures during harvest or at the calyx scar (Barkai-Golan, 2001). *Alternaria* mycotoxins that have been detected in moldy tomatoes include AOH, AME and TEA (Stack et al., 1985; Hasan, 1996).

Temperature is one of the major environmental factors that affect the shelf life of tomato fruits and their rate of deterioration by *A. alternata*. To control the growth and toxin production in tomato fruits, the temperature should be maintained below 7°C, and the storage period should not exceed 10 days (Hasan, 1996). Özcelik et al. (1990) studied the effects of packaging on formation of *Alternaria* toxins in intact tomatoes inoculated with *A. alternata* strain 8442–3. When compared to unpackaged tomatoes, the fruits shrink-wrapped with high-density polyethylene film contained lower toxin (TEA, AOH, AME, ALT) levels although the packaging did not completely inhibit fungal growth.

Direct consumption of moldy tomatoes by consumers is unlikely, but the possibility exists that these tomatoes could be used for processed tomato products (juice, puree, sauce, ketchup). To prevent mycotoxin contamination of processed tomato products, moldy or damaged tomatoes should not be used.

## 4.5. OCHRATOXIN A

### 4.5.1. GENERAL ASPECTS

Ochratoxin A (OTA) is a pentaketide mycotoxin produced by several *Aspergillus* and *Penicillium* species in different plant products such as cereals, coffee beans, cocoa beans, nuts, beer, wine, spices, vine fruits (raisins, currants and sultanas) (Aish et al., 2004; Varga and Kozakiewicz, 2006). OTA has been shown to be a carcinogen (Lock and Hard, 2004), and possess nephrotoxic (Mortensen et al., 1983), immunotoxic (Creppy et al., 1983), tetratogenic (Mayura et al., 1989) and genotoxic (Creppy et al., 1985; Obrecht-Pflumio et al., 1999) properties. It also has been implicated as the agent responsible for Balkan Endemic Nephropathy, a fatal kidney disease primarily affecting rural populations in the central Balkan peninsula (Vukelic et al., 1991; JECFA, 2001). The International Agency for Research on Cancer (IARC) has given OTA a Group 2B classification – a possible human carcinogen (IARC, 1993; Kuiper-Goodman and Scott, 1996). As a consequence of the possible health hazards related to ingestion of OTA, the Commission of the European Communities (EC) has set maximum levels for OTA in some foods including roasted coffee (5 µg/kg), instant coffee (10 µg/kg), raw cereal grains (5 µg/kg), processed cereal products (3 µg/kg), baby foods and processed cereal-based products for infants and young children (0.5 µg/kg), grape-based wines (2.0 µg/kg), grape juices (2.0 µg/kg) and dried vine fruits (10 µg/kg) (European Commission, 2005). There are also national laws and regulations in the member states covering other foods not regulated by European law. Of the products which are known to contain OTA, cereals are considered to

represent 50–80 percent intake of the toxin (SCOOP, 2002). Data on OTA occurrence in foods suggest that after cereals, wine is the major source of daily intake of OTA (Codex Alimentarius Commission, 1999; Battilani et al., 2006). Grape juice and raisins are also major sources of OTA in the diet, especially for young children.

#### 4.5.2. OCCURRENCE OF OTA IN FRUITS

There is considerable information on the natural occurrence of OTA in grape products including wine, juice, vinegar, raisins, dried sultanas (golden raisins) and dried currants (Drusch and Ragab, 2003; Battilani et al., 2006; Varga and Kozakiewicz, 2006). Since the first reports of OTA in wine (Zimmerli and Dick, 1996) several surveys have been conducted to assess the levels of the toxin in wine from Europe (Majerus and Ottener, 1996; Zimmerli and Dick, 1996; Miraglia and Brera, 2002; Domijan and Peraica, 2005), Australia (Majerus et al., 2000; Soleas et al., 2001; Hocking et al., 2003), Canada (Soleas et al., 2001; Ng et al., 2004), the United States (Ng et al., 2004) and South America (Majerus and Ottener, 1996; Soleas et al., 2001). Miraglia and Brera (2002) reported contamination levels as high as 15.6 µg/L in southern Europe. Among wines, red wines and sweet wines made from sun-dried grapes have the highest OTA contents (Bellí et al., 2002; Miraglia and Brera, 2002; Valero et al., 2005).

In general, the surveys indicate that OTA levels depend on the region in which the wine was produced. Wines produced in lower latitude regions (warmer climates) generally have higher incidence and concentrations of the toxin than those produced in higher latitude regions (cooler climates). In addition, OTA was found more frequently and in higher concentrations in European red wines than white wines (Ottener and Majerus, 2000; Battilani and Pietri, 2002; Miraglia and Brera, 2002). This trend was not apparent for wines produced in Australia; there were no differences in OTA levels in red vs. white wines (Leong et al., 2006). Similarly, Stefanaki et al. (2003) found no significant difference between OTA levels in red wines compared to white wines produced in Greece.

OTA can contaminate not only wine, but other grape products. Burdaspal and Legarda (1999) analyzed ten samples of grape juice and found OTA levels of 0.02–0.1 µg/L. Similarly, Wolff et al. (2000) examined samples of grape juice purchased in Germany between 1995 and 1998 and found that 7 out of 38 white grape juice samples contained less than 0.01 µg/L of OTA while the rest of the samples contained 0.01–1.3 µg/L. For the red grape juice samples, 8 out of 73 samples did not contain detectable OTA, while the rest had levels of 0.01–5.3 µg/L. Miraglia and Brera (2002) reported OTA levels as high as 5.3 µg/L in a German grape juice sample. More data on the occurrence and levels of OTA in grape juice is needed since children are major consumers of this product.

Vinegars are another grape product found to contain OTA. A survey by Majerus et al. (2000) has shown vinegar samples to contain 0.01–4.4 µg OTA/L with balsamic vinegar samples more frequently contaminated and with higher concentrations of OTA than regular vinegars. Similarly, Markaki et al. (2001) found that balsamic vinegars contained higher OTA levels (0.1–0.25 µg/L) than regular vinegar samples (<0.05 µg/L).

According to several surveys, OTA levels in dried grape products such as raisins, sultanas and currants are generally greater than the levels found in wine or grape juices. Levels of OTA in dried fruit in the range of 50–70 µg/kg were reported by Miraglia and Brera (2002). MacDonald et al. (1999) analyzed currants, raisins and sultanas purchased in the United Kingdom and found that 88 percent of samples contained OTA in the range of 0.2–53.6 µg/kg. However, on average, OTA levels in dried fruits are typically less than 3 µg/kg (MacDonald et al., 1999; Lombaert et al., 2004). OTA content of dried vine fruits has also been examined in the United States, and the average OTA levels were found to range from 0.42 µg/kg in 1997 to 1.27 µg/kg in 1998–1999 (Food and Agriculture Organization, 2001). It is evident that dried vine fruits can be highly contaminated with OTA, and thus be an important dietary source of OTA for people with high levels of consumption, in particular children.

### 4.5.3. FACTORS AFFECTING FORMATION OF OTA IN FRUIT

#### 4.5.3.1. Introduction

OTA is produced by a number of fungal species that can colonize a range of food products. They include *P. verrucosum* and *A. ochraceus*, fungi that predominantly infect cereal grains, coffee and cocoa. *P. verrucosum* is more important in cool, damp climates of northern Europe, while *A. ochraceus* is found in regions with warmer climates. However, neither of these OTA-producing fungi has been reported as the normal fungi affecting grape-derived foods. Members of the *A. niger* section *nigri* (black aspergilli), especially *A. carbonarius*, have been found to be the species responsible for colonization and OTA production in grape products (Abarca et al., 2003; Battilani et al., 2006). These species are common soil inhabitants whose spores become airborne and contaminate ripening crops in Mediterranean, tropical and subtropical regions (Magan and Aldred, 2005).

Black aspergilli are the most common fungi responsible for post-harvest decay of fresh fruits and are found on the surface of healthy grapes at all stages of development (Pitt and Hocking, 1997; Zahavi et al., 2000). They are causal agents of several plant diseases, are opportunistic pathogens of grapes, and cause bunch rot or berry rots and raisin mold (Varga and Kozakiewicz, 2006). As opportunist pathogens, these species attack fruit through skin breaks or from damage to vines. Proliferation of the fungus and OTA formation in grapes used in dried fruit production is believed to occur when fruits are harvested and during drying.

The presence of OTA as a contaminant of grape products is dependent on microclimate conditions which facilitate germination, germ tube extension, establishment and mycelial colonization to occur (Magan and Aldred, 2005). At present, the ecological parameters of black aspergilli are not completely known, but the most important factors affecting the life cycle of the fungus include  $a_w$ , temperature, and chemical properties of the food matrix (Bellí et al., 2004; Mitchell et al., 2004; Bellí et al., 2005). In general, the optimum temperatures for growth of *A. carbonarius* strains are 30–35°C with minimal growth at temperatures less than 15°C. The growth rate of the fungus increases with increasing  $a_w$ , with the maximum growth rate being between 0.93 and 0.99 $a_w$  (Mitchell et al., 2004). OTA production is also favored by high  $a_w$  levels, and although it was observed in the whole range of temperatures, maximum amounts were produced by *A. carbonarius* strains at 20°C (Bellí et al., 2005) and *A. niger* strains at 20–25°C (Esteban et al., 2004). OTA is produced rapidly by fungal colonies (Battilani et al., 2006). Maximum amounts of OTA were found in 5 days by *A. carbonarius* strains and in 7–13 days by *A. niger* strains (Bellí et al., 2004).

#### 4.5.3.2. Pre-harvest conditions

The effect of pre-harvest conditions on OTA formation in grape-derived foods has been studied in detail, while little is known about how these conditions affect toxin production in other foods. Pre-harvest factors that are believed to affect OTA levels in wine and other grape-derived products include location of the vineyard, weather conditions, cultivar of grape and vineyard management. Wines and juices produced in countries in the Mediterranean, including southern regions of France and Italy, Greece and certain regions of Portugal and Spain, are particularly susceptible. These trends can be explained by the climatic differences between geographical regions which, in turn, affect mold contamination and OTA production (Abrunhosa et al., 2001; Serra et al., 2003). The importance of weather conditions on OTA formation was illustrated by Pietri et al. (2001) and Lopez de Cerain et al. (2002). OTA-producing aspergilli favor regions characterized by hot and dry summers rather than temperate or cool climates. Grape varieties have been shown to vary in their susceptibility of OTA accumulation (Battilani et al., 2004).

Rousseau and Blateyron (2002) indicated that the occurrence of OTA in wine may be decreased substantially by using appropriate vineyard management. Management of black aspergilli in vineyards should focus on the health of the berries between early veraison and ripening, and on decreasing the incidence of black aspergilli in the vineyard (Battilani et al., 2004). Leong et al. (2006) outlined practices for managing *A. carbonarius* in vineyards including producing small, loose bunches of grapes that are well dispersed on the vine, preventing pest damage to berries and bunches, especially between veraison and harvest, minimizing mechanical and environmental damage to berries, and minimizing incidence of *A. carbonarius* in bunches by keeping soil free of decaying plant debris.



Preventing the growth of ochratoxigenic fungi is believed to be the most effective strategy for controlling the presence of OTA in foods. Attempts have been made to reduce fungal contamination of grapes through the use of fungicides. Belli et al. (2006) studied the impact of fungicides on *A. carbonarius* growth and OTA production. In a synthetic medium and on grapes, fungicides that contained copper or strobilurins reduced both growth of the fungus and OTA production. Temperature also influenced the effectiveness of the fungicides. This work is in accordance with a previous study by Lo Curto et al. (2004), who found that application of pesticides in combination with fungicides were effective at decreasing OTA levels of wines. Biological control through the use of *Bacillus thuringiensis* was found to be effective at reducing the growth of OTA-producing fungi on grapes (Bae et al., 2004). More research is needed to identify effective treatments for preventing infection with *A. carbonarius* and OTA production in vineyards.

#### 4.5.3.3. Harvest and post-harvest conditions

Health or condition of fruits at harvest or post-harvest profoundly influences OTA accumulation. Rotten grape berries or those damaged by insects, fungal pathogens, excessive irrigation or rain damage, or during harvest should be avoided for production of wines since the black aspergilli proliferate and produce OTA in the presence of nutrients from the grape flesh (Varga and Kozakiewicz., 2006). Culling or removing damaged and moldy grapes prior to crushing and pressing or to drying is effective in reducing OTA levels in wine, juice, vinegar and dried fruit products (Kozakiewicz et al., 2003).

Little information is available on the effects of the steps used in winemaking on the fate of OTA in wine. The observation that red wines tend to be more highly and frequently contaminated with OTA than white wines has led to speculation that processing differences for the two types of wine may be responsible (Majerus et al., 2000). The relatively longer mash standings used in the manufacture of red wines compared to that of white or rosé wines may result in proliferation of ochratoxigenic aspergilli and accumulation of OTA (Majerus et al., 2000). OTA does not appear to be produced during the fermentation of grape juice into wine, mainly because alcohol inhibits fungal growth (Delage et al., 2003). However, OTA present in grape juice persists during fermentation (Zimmerli and Dick, 1996). Processing treatments such as use of bacteria (*Lactobacillus plantarum* and *Oenococcus oeni*) or absorbents such as charcoal, liquid gelatin or yeast cell wall preparations have been shown to bind OTA and reduce OTA levels in wines (Castellari et al., 2001; Varga and Kozakiewicz, 2006).

Processing of grapes into dried fruit products has been shown to have a substantial effect on OTA levels in the final consumer products. Fruits to be used for drying must be of high quality and the process of drying must start immediately after harvest (Magan and Aldred, 2005). Fruits are generally sun-dried for 7–14 days and turned regularly to control moisture loss. During



the process, sugars in the fruit become more concentrated resulting in a selective medium for black aspergilli. The occurrence of rain during the drying process increases the risk of OTA contamination since drying becomes uneven. Consequently, the drying process should be done as rapidly as possible and the product packaged and stored only after proper moisture levels have been reached (Drusch and Ragab, 2003).

## 4.6. CONCLUSIONS

Fungal spoilage of fruits causes substantial financial losses to fruit growers and processors and may pose a health threat to the consumer if the contaminating fungi produce mycotoxins. Mycotoxins most commonly associated with fruits are patulin, aflatoxins, ochratoxin A and *Alternaria* toxins. The extent of fungal growth and subsequent mycotoxin production depends on the type and cultivar of fruit, the physical and chemical properties of the fruit, the ripening state of the fruit, the microbial load and the presence of surface defects. Temperature and relative humidity are environmental factors that influence fungal growth and mycotoxins production. Since mycotoxin accumulation in fruits can occur in the field, at the time of harvest, post-harvest and during storage, the best approach to prevent contamination is to prevent mold growth at all stages of production. Research to date indicates that an integrated approach including careful handling of fruit to prevent skin breaks and structural damage and use of sanitary conditions in the fields or orchards, storage facility and processing facilities is essential for reducing fungal decay and mycotoxins production in fruits. Although consumers will likely reject fresh fruit that is visibly moldy or rotten, processed fruit products may contribute significant amounts of these toxins if decayed or moldy fruit is not removed before processing or packaging. Thus, only sound fruit should be used for processed fruit products. Fruits such as figs, dates or grapes that will be used in dried fruit production, should be dried rapidly and completely, and stored under conditions that prevent rewetting.

Surveys are needed to determine the levels of aflatoxins in dried fruits, *Alternaria* toxins in processed tomatoes and fruit products, and OTA in grape products. In addition, studies are needed to identify fruit cultivars that are resistant to fungal decay and to evaluate treatments (e.g., fungicides, biological antagonists, heat treatments) that could be used to control fungal pathogens in the field or post-harvest. Finally, more work is needed to determine the fate of mycotoxins during processing.

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# Diffusion of Mycotoxins in Fruits and Vegetables

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## ABSTRACT

This chapter reviews papers describing mycotoxin migration from rotten to unaffected parts of fruits and vegetables. It is known that mycotoxins can remain in fruits even when the fungal mycelium producing them has been eliminated and, depending on the characteristics of the fruit, mycotoxins can diffuse into sound tissues. Since second-quality fruits may be used to produce derivatives such as juices, jam, jelly, yoghurt, fruit salad, etc., a study of mycotoxin behavior in affected fruits is important to establish suitable means of protecting consumers from exposure to highly toxic substances such as ochratoxin A and patulin.

Mycotoxin diffusion from moldy to unaffected parts of fruit and vegetables is reviewed in relation to: (a) the species of contaminating fungus; (b) the size of the rotten area; (c) the distance from the affected area and (d) the characteristic consistency and/or composition of fruit or vegetable pulp.

## 5.1. INTRODUCTION

That mycotoxins can be present in fruits and vegetables is well known – see elsewhere in this book. In this chapter, factors affecting the diffusion of mycotoxins from the rotten to the unaffected area of fruits is discussed. This is particularly critical for the safety of consumers since parts of rotten fruits/vegetables that are not rotten can be used in the preparation of fruit salad, juices, yoghurt, jam or jellies. It has been shown that contamination of fruit juices by mycotoxins results mainly from the use of poor-quality fruits.

Of the large number of known mycotoxins, only a few are commonly found in fruits and vegetables: aflatoxins (produced by several *Aspergillus* species), ochratoxin A (by *Penicillium* and *Aspergillus* species), alternariol and derivatives (by *Alternaria* species), patulin (by *Penicillium* and *Aspergillus* species), citrinin (by *Penicillium expansum*) and trichothecin (by *Trichothecium roseum*) (Drusch and Ragab, 2003).

Little has been published about the migration of mycotoxins in fruits and vegetables; and in those few publications this aspect was not always a primary goal of the research. This review will be organized along the following lines:

- The role of fruit skin integrity in mycotoxin diffusion
- Evidence for mycotoxin diffusion from rotten to unaffected areas
- Factors affecting mycotoxin diffusion in fruits and vegetables.

## 5.2. ROLE OF FRUIT SKIN INTEGRITY IN MYCOTOXIN DIFFUSION

A paper by Buchanan et al. (1975) concerning figs indicates that conidia of *Aspergillus flavus* may have penetrated fig skin even when the fruits do not present evident wounds. These authors showed that green fruits are resistant to attack by *A. flavus* but when they become ripe they lose this resistance because the skin is soft. Spores on the surface of ripe fruits were shown to infect and colonize the fruits, leading the authors to deduce that conidia can penetrate the skin even when it is intact. However, there might have been other causes for the penetration, the most probable being the presence of microscopic organisms such as insects or mites, which can produce minute skin wounds not visible to the eye, or the presence of juice exuded onto the fruit surface that allowed fungal colonization and mycotoxin penetration. Rapid fungal colonization and aflatoxin production continue until the growth of *A. flavus* is halted by adverse environmental conditions, such as the lack of moisture in dried fruit (Buchanan et al., 1975).

In contrast to figs, oranges are contaminated by *A. flavus* only after the peel has been visibly attacked by other microorganisms, probably because citrus oil has antifungal properties (Varma and Verma, 1987).

## 5.3. EVIDENCE FOR MYCOTOXIN DIFFUSION FROM ROTTEN TO UNAFFECTED AREAS

The diffusion of mycotoxins, mainly ochratoxin A and patulin, from a moldy area of a fruit to unaffected parts has been studied by various authors. The results (Table 5.1) indicate that damaged or moldy fruits can be contaminated with mycotoxins to some degree even after the rotten area has been removed.

Engelhardt et al. (1999) analyzed different fruits after the removal of rotten tissue and found up to 2.71 µg/kg of ochratoxin A in cherries and up to 1.44 µg/kg in tomatoes and strawberries, while peaches and apples were contaminated to a lesser degree (0.59 and 0.41 µg/kg, respectively).

TABLE 5.1 Mycotoxin concentration in fruits after removal of rotten area.

Mycotoxin	Fruit considered	Maximum mycotoxin concentration detected (µg/kg)	Reference
Ochratoxin A	Apple	0.41	Engelhardt et al., 1999
	Apricot	ND	
	Cherry	2.71	
	Nectarine	ND	
	Peach	0.59	
	Strawberry	1.44	
	Tomato	1.44	
Patulin	Apple (with peel)	1166	Beretta et al., 2000
	Apple (without peel)	93	
	Pears	288 000 <sup>a</sup>	Laidou et al., 2001

ND = not detectable.  
<sup>a</sup>inoculated with *Penicillium expansum*.

Since patulin is one of the most frequently detected mycotoxin in fruits and their derivatives, most papers considered the migration of this mycotoxin in fruits and vegetables. Beretta et al. (2000) measured the migration of patulin in 26 apples with different-sized rotten areas containing active molds. To investigate whether the mycotoxin could migrate to areas not affected by rot, two identical portions of each naturally affected apple were analyzed after the removal of the rotten area, one with and one without the peel.

Five out of 26 apples did not contain the mycotoxin, confirming that some apple molds are not capable of producing patulin, if the environmental conditions are not right. Figure 5.1 shows data from the remaining 21 moldy apples. The mycotoxin concentration in the rotten area was often very high: up to 113.3 mg/kg. Mycotoxins were detected in the unaffected portions of 17 out of 21 samples when the peel was not removed and 7 apples still presented detectable quantities of patulin after peeling. The content of patulin in unpeeled portions ranged from 0.05 to 1166 µg/kg, while that of the peeled part was between 0.44 and 93 µg/kg (Table 5.1 and Fig. 5.1). These data, and those concerning apple juices published by the same authors, show that if apple derivatives are prepared with low-quality fruits, the concentration of patulin could exceed the safe limits established by the international toxicological committees (50 µg/kg or µg/l). In fact, commercial apple juices containing pulp analyzed by these authors showed a patulin concentration of 0.68–1150 µg/kg (Beretta et al., 2000).

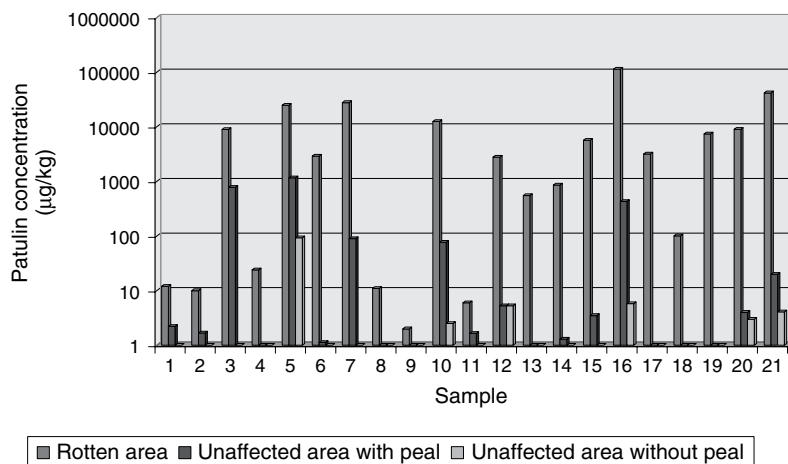


FIGURE 5.1 Patulin concentration ( $\mu\text{g/kg}$ ) in the affected and unaffected areas of 21 rotten apples. Values lesser than were changed to 1  $\mu\text{g/kg}$  in order to obtain a zero on the semilogarithmic scale. Data from Beretta et al., 2000.

## 5.4. FACTORS AFFECTING MYCOTOXIN DIFFUSION IN FRUITS AND VEGETABLES

### 5.4.1. PATULIN MIGRATION AND SIZE OF THE ROTTEN AREA

A common finding by different authors is that there is no direct correlation between the patulin concentration in apple/apple derivatives and the percentage of rottenness in the fruits (Beretta et al., 2000; Martins et al., 2002). In some cases, very high contents of patulin were detected in apples with a small affected area (Fig. 5.2) (Beretta et al., 2000). For instance, apple no. 5 showed 6.4 percent of rottenness but a very high content of patulin ( $93 \mu\text{g/kg}$ ), also in the peeled unaffected area; by contrast, apple nos. 20 and 21, in which 50 percent of the apple was affected, contained only a few  $\mu\text{g/kg}$  of mycotoxin in the sound part of the fruit.

### 5.4.2. PATULIN MIGRATION CAN BE AFFECTED BY THE CONSISTENCY OF THE FRUITS

Several authors have studied the diffusion of patulin from the rotten to unaffected areas. Some of them (Ostry et al., 2004; Rychlik and Schieberle, 2001; Taniwaki et al., 1992) found the diffusion of patulin in apples to be limited to a depth of 1–2 cm from the rotten area.

Taniwaki et al. (1992) studied the migration of patulin in 22 apples, in an attempt to determine how much trimming of the rotten portion of fruit is needed to avoid the contamination of derivatives. The authors inoculated

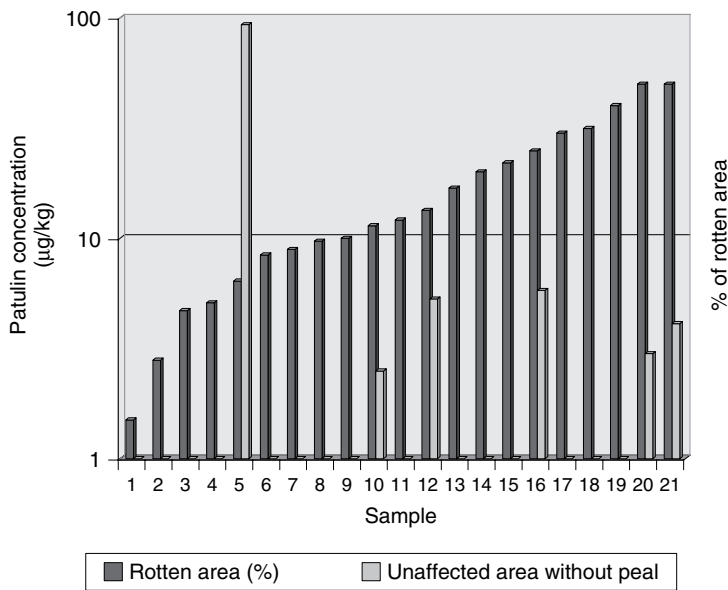


FIGURE 5.2 Patulin concentration ( $\mu\text{g/kg}$ ) in the unaffected area without peel versus size of rotten area (% of total fruit). Patulin concentrations  $<1$  were changed to  $1 \mu\text{g/kg}$  in order to obtain a zero on the semilogarithmic scale. Data from Beretta et al., 2000.

apples with 5-day-old mycelia of patulin-producing *Penicillium expansum*, inserting a 3 mm needle into each apple. The apples were then incubated at  $25^{\circ}\text{C}$  until the diameter of the lesion reached 3.6–4.8 cm. After the development of lesions, the moldy area was removed with circular knives having a diameter depending on the extent of mold growth (3.6–4.8 cm in diameter and 10 cm in length). The cylinder obtained was then divided into 6 portions 1 cm thick, and their patulin content was measured (Fig. 5.3).

The data from the same authors illustrated in Figs 5.4 and 5.5 show that:

- the extent of the lesions (internal diffusion) in the 22 apples inoculated with *P. expansum* varies widely, even though the diameter of the superficial moldy areas was similar (3.6–4.8 cm);
- the presence of lesions does not imply the presence of patulin; for example, at 3 cm from the inoculation point 41 percent of the apples showed a lesion, but only 2 out of 9 contained a detectable amount of patulin;
- in portions with no lesion or even close to a lesion, patulin was undetectable or present at low concentration;
- most of the patulin present in the apple tissue was found 1 cm from the patulin containing lesion.

The authors concluded from these data that contamination of apple derivatives could be avoided by trimming and removing a 1 cm portion around and under the rotten area.



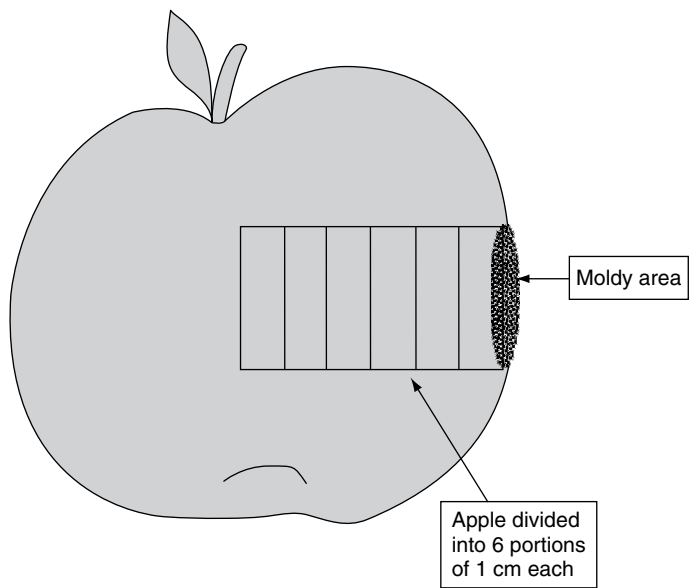


FIGURE 5.3 Removal of apple portions to detect lesion extent and patulin migration (modified from Taniwaki et al., 1992).

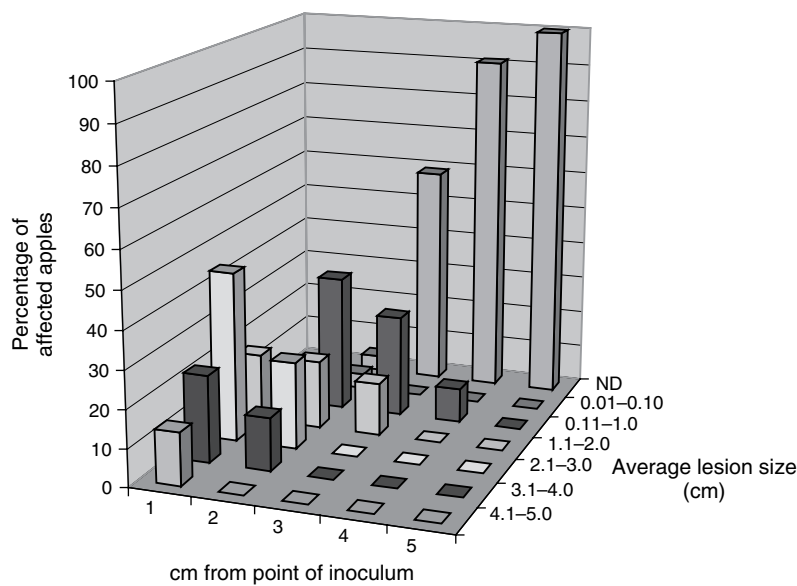


FIGURE 5.4 Extent of lesions in six portions of apples inoculated with *Penicillium expansum*: percentage of affected apples versus distance from the inoculation point. (ND = undetectable) Data from Taniwaki et al., 1992.

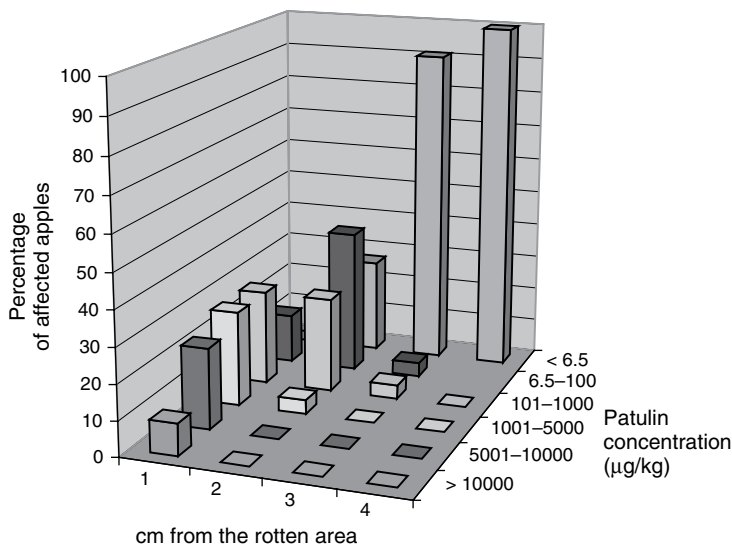


FIGURE 5.5 Migration of patulin in apples inoculated with *Penicillium expansum* and percentage of affected apples at different distances from the rotten area. Data from Taniwaki et al., 1992.

Figure 5.6 shows the results of Rychlik and Schieberle (2001), who examined patulin migration in different matrices, and revealed that the risk of mycotoxin diffusion is dependent on the consistency of the fruit or vegetable concerned. Migration was studied on apples, tomatoes, and bread experimentally infected by *Penicillium expansum*, and the extent of diffusion was examined after 7 days of incubation. Starting from the rotten area, which was

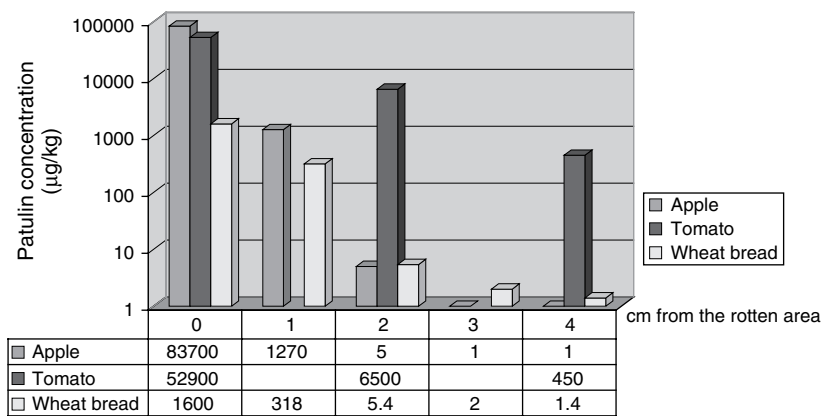


FIGURE 5.6 Patulin concentrations (µg/kg) at different distances from the rotten area of apple, tomato, and wheat bread crumbs infected by *Penicillium expansum*. Mycotoxin concentration in apples at 3 and 4 cm were  $< 6 \times 10^{-2}$  µg/kg; they were changed into 1 µg/kg in order to obtain a zero on the semilogarithmic scale. Data from Rychlik and Schieberle, 2001.

scraped out, samples were cut in spherical segments 1 cm thick and patulin was quantified in each section. In apples with an initial concentration of 83.7 mg/kg in the affected tissue, the mycotoxin concentration rapidly dropped with distance, down to  $<0.0012$   $\mu\text{g/kg}$  at 3 cm from the rotten area.

Unlike apples, tomatoes were penetrated easily by patulin: at 4 cm from the moldy tissue the concentration of patulin was still 0.450 mg/kg.

After infection of wheat bread with *Penicillium expansum* and incubation for 7 days, the concentration of patulin in the layer containing fungal mycelium was 1.6 mg/kg; there was little diffusion and the contamination dropped sharply off with distance and was barely detectable 4 cm from the affected area. The authors concluded that, like “viscous” apple tissue, bread has also a consistency which reduces patulin diffusion.

These results indicate that the consistency of the matrix is crucial in determining mycotoxin diffusion, which is facilitated by high water content and hindered by the presence of structure-forming polysaccharides. Thus, it has been shown that patulin penetrates more easily in water-rich fruits such as grapes, melons, tomatoes, and blueberries, while viscous matrices like those of apples and bread restrict patulin migration to a few centimeters from the rotten area.

#### 5.4.3. PATULIN DIFFUSION IN FRUITS DEPENDS ON THE PARTICULAR PATHOGEN

Laidou et al. (2001) considered the diffusion of patulin in the flesh of pears after infection with four different pathogens: *Penicillium expansum*, *Aspergillus flavus*, *Stemphylium vesicarium*, and *Alternaria alternata*. The inoculated fruits were incubated at room temperature (18–20°C) for 2–22 days, depending on the strain inoculated. The infected fruits were then split longitudinally through the center of the lesion (5–20 mm in diameter) and were divided into four parts, from the tissue immediately below the lesions to the area around the carpel. These authors showed that the isolation frequency of all the fungi increased in all the sections as the lesion diameter increased. This was considered compatible with other data such as those by Thanassouloupoulos et al. (1990), who demonstrated a correlation between lesion diameter and the depth of penetration of *Alternaria alternata* in pears. *Penicillium expansum* and *Aspergillus flavus* showed higher frequencies of isolation at all depths than did *Stemphylium vesicarium* and *Alternaria alternata*. The two former fungi also showed more rapid penetration into the flesh; on the other hand, *P. expansum* and *A. alternata* penetrated the fruit flesh before there was any patulin diffusion, while the penetration of *S. vesicarium* lagged behind the diffusion of patulin.

Patulin was detected in the flesh of pears in areas of 10–20 mm diameter from the rotten area when the fruit was inoculated with *P. expansum*, *S. vesicarium* or *A. alternata*, but not with *A. flavus*. The mycotoxin was detected even in apparently sound tissues (Fig. 5.7).

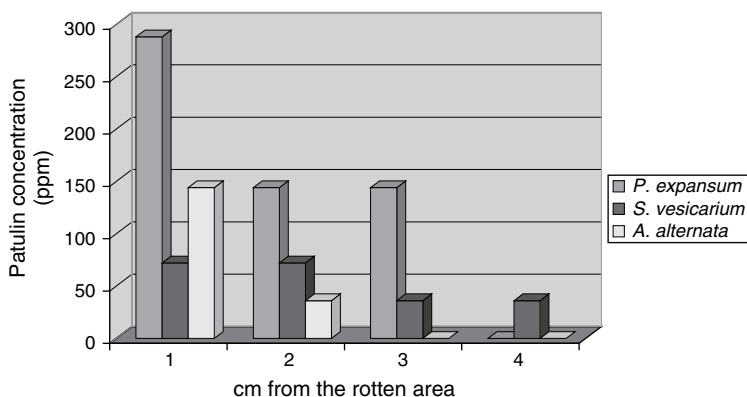


FIGURE 5.7 Patulin concentration (mg/kg) at different depths of pears inoculated with *Penicillium expansum*, *Stemphylium vesicarium*, and *Aternaria alternata*. Data from Laidou et al., 2001.

## 5.5. CONCLUSIONS

The question raised by this short review is, what is the risk for consumers if a food manufacturer uses partially rotten fruits to derive a product, even if affected portions have previously been removed?

The above data suggest several strategies to ensure safety in the use of fruits with limited moldy area(s):

- the consistency of the fruit matrix should be considered: moldy fruits having a high water content and containing few structural polysaccharides must be avoided;
- cutting off the rotten tissue up to a distance of 2 cm from the mold seems enough to remove most of the hazardous mycotoxin in most situations;
- up to 99 percent of patulin can be eliminated by removing the moldy area and then suitably washing the fruit, e.g., by high-pressure water spraying (Acar et al., 1998; Lovett et al., 1974) – the initial patulin concentration in apples can be reduced 10-fold by combining washing and removal of the damaged area (Leggott et al., 2000);
- patulin concentration can be significantly reduced by clarification (Gökmen et al., 2001) or ultrafiltration processes (Acar et al., 1998).

Although these strategies can improve the quality of derivatives from second-quality fruits, the procedures are not easily achieved, and careful selection of fruit quality still remains the most appropriate procedure to protect consumers' health. Moreover, elimination of the rotten area must include an unaffected area large enough to reduce the patulin level below 50 µg/kg, and this is not easily attained by automated processes. As shown by different authors, the use of second-quality fruit is acceptable only for the production of clear juices, in which the filtration process eliminates most of the mycotoxin with the fruit pulp.

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# Aspergillus Mycotoxins

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## ABSTRACT

Aflatoxins are often found in fruits of tropical and subtropical regions, where typical environmental conditions support growth of aflatoxigenic aspergilli, and mycotoxin production by them. Special attention has been drawn to aflatoxin production by *Aspergillus flavus* and *A. parasiticus* in figs and dates. Figs are vulnerable to infection during their drying, but before they are dried completely. Dates grown under high humidity and moderate temperatures are especially exposed to aflatoxin contamination during their later stages of maturation. In contrast to figs, date contamination is not associated with dried fruits. The absence of aflatoxins in the early stages of date maturation suggested that these fruits are initially free of aflatoxins.

Ochratoxin A (OTA) in dried figs has been related to the presence of *A. alliaceus*. Use of a sensitive methodology for OTA extraction from dried figs enabled high levels of OTA to be found, and co-occurrence of aflatoxin B<sub>1</sub> and OTA was frequently recorded.

Until 1996 *A. ochraceus* was regarded as the only *Aspergillus* responsible for OTA production in grapes. However, extensive studies, especially in the Mediterranean area, led to the conclusion that, although several aspergilli may naturally infect grapes, *A. carbonarius* is the key fungus responsible for ochratoxin contamination in wines and other grape-based products. Other ochratoxigenic *Aspergillus* spp. belonging to section Nigri, *A. tubingensis*, and, to a lesser extent, *A. niger* may also contribute to the occurrence of OTA in wines, along with the major contribution of *A. carbonarius*. The effects of fruit maturity and environmental conditions on OTA production in grapes, and the possible interactions of ochratoxigenic aspergilli with other fungi present on the berries during maturation have been studied.

Other *Aspergillus* mycotoxins, that are associated with fruits and vegetables, such as cyclopiazonic acid, sterigmatocystin, aflatrem, citrinin and penicillic acid, and the co-occurrence of different mycotoxins in fruit and fruit-derived products, have been discussed.

## 6.1. INTRODUCTION

*Aspergillus* species are widespread in nature. They are regarded as soil fungi, frequently colonizing plant debris and decaying agricultural crops, and are

among the commonest airborne fungi (Gregory, 1973). They occur with high frequency as saprophytes on a wide range of substrates, including foods and feeds, and several species are among the typical pathogens of harvested fruits and vegetables (Barkai-Golan, 1980; Snowdon, 1990; Barkai-Golan, 2002).

During their life cycle, several *Aspergillus* species are capable of producing a wide range of mycotoxins harmful to humans and animals that consume them (Moss, 1977; Scott, 1994, 2004). In fact, mycotoxin production by aspergilli is a most important consequence of fruit and vegetable infection. The major mycotoxins associated with *Aspergillus* species in fruits and vegetables are aflatoxins produced mainly by aflatoxigenic strains of *A. flavus* and *A. parasiticus* in figs and dates (Doster et al., 1996; Shenasi et al., 2002) and ochratoxin A (OTA) produced by *A. carbonarius* and other ochratoxigenic aspergilli in grapes, dried vine fruits and wines (Battilani and Pietri, 2004).

Although OTA and aflatoxins are the most important *Aspergillus* mycotoxins in fruits, other mycotoxins synthesized by aspergilli, such as cyclopiazonic acid, sterigmatocystin, citrinin, penicillic acid, aflatrem and other secondary metabolites, may also elicit toxicological effects.

The classic guide to the taxonomy of *Aspergillus* is *The Genus Aspergillus* by Raper and Fennell (1965), which is based mainly on cultural and morphological features. New taxonomic approaches followed by taxonomic revisions and new species have been introduced by Frisvad (1986), Klich and Pitt (1988), Kozakiewicz (1989) and Pitt and Hocking (1997). Since the discovery of OTA in grape juices and, especially, in wines (Zimmerli and Dick, 1996) the interest in mycotoxin production by *Aspergillus* species has markedly increased. This has led to the need for an accepted taxonomy of this genus that includes toxicological aspects (Wilson et al., 2002). Molecular approaches have contributed to the taxonomy of *Aspergillus* by showing the variability in the complex group of black aspergilli from the genetic point of view (Ménégneau et al., 1993) and by clarifying taxonomic relationships among toxigenic aspergilli contaminating agricultural products (Varga et al., 2004).

## 6.2. POSTHARVEST MYCOTOXIGENIC *ASPERGILLUS* SPECIES AND THEIR TOXINS

Some of the mycotoxins produced by *Aspergillus* species during their developmental stages in fruits and vegetables are among the most important carcinogenic mycotoxins; they include aflatoxins, ochratoxin A (OTA) and sterigmatocystin.

*Aspergillus* mycotoxins in fruits can be divided into the following three major groups: (1) Aflatoxins produced by *A. flavus*, *A. parasiticus* and *A. nomius*; (2) Ochratoxin A produced by *A. ochraceus*, *A. carbonarius*, *A. niger* aggregate, *A. tubingensis*, *A. sclerotiorum*, *A. sulphureus*, *A. aculeatus*, *A. japonicus* var. *aculeatus*, *A. alliaceus*, *A. melleus* and other species; and (3) Other toxic metabolites produced by a variety of *Aspergillus* spp., the most important of these being sterigmatocystin, produced by *A. flavus*, *A. flavipes*, *A. nidulans* and *A. versicolor*; cyclopiazonic

acid, produced by *A. flavus*, *A. tamarii* and *A. versicolor*; aflatrem, produced by *A. flavus*; citrinin, produced by *A. flavipes*, *A. carneus*, *A. niveus* and *A. terreus*; and patulin, produced by *A. terreus*.

Mycotoxigenic *Aspergillus* species associated with postharvest diseases of fruits and vegetables, and mycotoxins produced by them, are listed in Table 6.1.

TABLE 6.1 Mycotoxigenic *Aspergillus* species associated with fruit infection, and secondary metabolites they are capable of producing.

<i>Aspergillus</i> Species	Mycotoxins and other secondary metabolites	References
<i>A. alliaceous</i>	Ochratoxin A	Doster et al., 1996; Bayman et al., 2002
<i>A. aculeatus</i>	Emodin	Semeniuk et al., 1971; Sage et al., 2002
	Ochratoxin A	Turner and Aldridge, 1983
	Secalonic acid D	Sage et al., 2002
<i>A. amstelodami</i>	Catenarin	Turner and Aldridge, 1983
<i>A. carbonarius</i>	Ochratoxin A	Battilani and Pietri, 2002; Cabañes et al., 2002; Sage et al., 2002; Serra et al., 2003
<i>A. chevalieri</i>	Gliotoxin	Vesonder et al., 1988; Richard et al., 1999;
<i>A. flavipes</i>	Sterigmatocystin	Davis, 1981
	Citrinin	Gorst-Allman and Steyn, 1979
<i>A. flavus</i>	Aflatoxins	Buchanan et al., 1975; Doster et al., 1996;
	Aflatrem	Giridhar and Reddy, 2001; Roussos et al., 2006
	aspergillic acid	
	Sterigmatocystin	Moss, 1977; Wicklow and Cole, 1982
	Cyclopiazonic acid	Cole and Cox, 1981
	Versicolorins	Davis, 1981
	Kojic acid	Gallagher et al., 1978; Doster et al., 1996
		Betina, 1989
		Moss, 1977
<i>A. foetidus</i>	Ochratoxin	Magnoli et al., 2004
<i>A. fumigatus</i>	Fumagillin	Cole and Cox, 1981
	Fumigatin	Cole and Cox, 1981
	Verruculogen	Moss, 1977
	Gliotoxin	Giridhar and Reddy, 2001
	Kojic acid	Cole and Cox, 1981
<i>A. glaucus</i> group	Erythroglaucin	Anke et al., 1980
	Emodin	Anke et al., 1980
	Questin	Anke et al., 1980
	Physcion	Anke et al., 1980
	Rubrocristin	Anke et al., 1980
	Anthraquinones	Anke et al., 1980
	Catenarin	Turner and Aldridge, 1983
<i>A. japonicus</i> var. <i>aculeatus</i>	Ochratoxin A	Magnoli et al., 2004
<i>A. melleus</i>	Ochratoxin A	Hesseltine et al., 1972; Doster et al. 1996; Bayman et al., 2002
	Xanthomegnin	Durley et al., 1975
	Viomellein	Durley et al., 1975
<i>A. nidulans</i>	Sterigmatocystin	Mislivec et al., 1975

(Continued)



TABLE 6.1—(Cont'd)

<i>Aspergillus</i> Species	Mycotoxins and other secondary metabolites	References
<i>A. niger</i>	Malformins	Moss, 1977; Kim et al., 1993
	Ochratoxin A	Moss, 1977; Iamanaka et al., 2005
<i>A. niger</i> var. <i>awamori</i>	Ochratoxin A	Magnoli et al., 2004
<i>A. niveus</i>	Citrinin	Raper and Fennell, 1965
<i>A. nomius</i>	Aflatoxins	Kurtzman et al., 1987; Moss, 2002
<i>A. ochraceus</i>	Ochratoxin A	Doster et al. 1996; Bayman et al., 2002; Ciegler, 1972; Hesseltine et al., 1972
	Xanthomegnin	Stack and Mislivec, 1978
	Viomellein	Stack and Mislivec, 1978
	Vioxanthin	Scudamore et al., 1986
	Malformins	Betina, 1989
	Penicillic acid	Ciegler, 1972
	Secalonic acid D	Turner and Aldridge, 1983
<i>A. parasiticus</i>	Aflatoxins	Doster et al., 1996
	Versicolorins	Lee et al., 1975
<i>A. sclerotiorum</i>	Ochratoxin A	Hesseltine et al., 1972; Doster et al., 1996; Varga et al., 1996; Bayman et al., 2002
<i>A. sulphureus</i>	Ochratoxin A	Semeniuk et al., 1971; Varga et al., 1996
	Xanthomegnin	Durley et al., 1975
	Penicillic acid	Gill-Carey, 1949
	Rubrosulphin	Cole and Cox, 1981
	Viomellein	Durley et al., 1975
<i>A. sydowi</i>	Sterigmatocystin	Davis, 1981
<i>A. tamarii</i>	Cyclopiazonic acid	Dorner et al., 1984; Doster et al., 1996
<i>A. terreus</i>	Citreoviridin	Cole and Cox, 1981
	Citrinin	Cole and Cox, 1981
	Patulin	Turner and Aldridge, 1983
	Terreic acid	Cole and Cox, 1981
	Terrein	Cole and Cox, 1981
	Gliotoxin	Turner and Aldridge, 1983
<i>A. tubingensis</i>	Ochratoxin A	Medina et al., 2005; Perrone et al., 2006
<i>A. ustus</i>	Sterigmatocystin	Davis, 1981
	Austocystin	Moss, 1977
	Austin	Cole and Cox, 1981
<i>A. versicolor</i>	Sterigmatocystin	Sommer et al., 1976; Moss, 1977
	Versicolorins	Lee et al., 1975
	Cyclopiazonic acid	Moss, 1977; Cole, 1984; Dorner et al., 1984
<i>A. wentii</i>	Ochratoxin A	Varga et al., 1996
	Emodin	Turner and Aldridge, 1983

## 6.3. AFLATOXINS

### 6.3.1. THE MAJOR AFLATOXINS AND THEIR TOXIC EFFECTS

Studies in the 1960s showed that aflatoxin splits into four main components when chromatographed on thin-layer silica gel plates (Hartley et al., 1963).

These were designated aflatoxins B<sub>1</sub> (C<sub>17</sub>H<sub>12</sub>O<sub>6</sub>) and B<sub>2</sub> (C<sub>17</sub>H<sub>14</sub>O<sub>6</sub>), which fluoresced blue under UV light; and G<sub>1</sub> (C<sub>17</sub>H<sub>12</sub>O<sub>7</sub>) and G<sub>2</sub> (C<sub>17</sub>H<sub>14</sub>O<sub>7</sub>), which fluoresced turquoise under UV light. The isolation of the four main aflatoxins was followed by discoveries of numerous other analogues of aflatoxins. Of the four major aflatoxins, aflatoxin B<sub>1</sub> has the greatest acute toxicity to animals followed by G<sub>1</sub>, B<sub>2</sub> and G<sub>2</sub> (Heathcote, 1984). Aflatoxin B<sub>1</sub> was recognized as the most carcinogenic type (Baird et al., 2006): it causes malignant tumors in various animals, the liver being the major target organ. It was considered as a group I carcinogen by the International Agency for Research on Cancer, as being the cause of human primary hepatocellular carcinoma (IARC, 2002).

The carcinogenic and mutagenic actions of aflatoxins at the biochemical and molecular levels are both associated with their DNA binding properties, whereas acute toxicity may also be associated with aflatoxin-protein binding. Aflatoxin B<sub>1</sub> has also teratogenic properties and causes malformations in many organs in embryos of various mammals, birds, amphibians and fish. Since teratogenicity is associated with mutations and gross alteration in the structure of chromosomes, the DNA-binding property of aflatoxin B<sub>1</sub> may be the key reaction (Stark, 2001).

Aflatoxins B<sub>1</sub> and G<sub>1</sub> are the main aflatoxins that occur in agricultural commodities.

### 6.3.2. FUNGI ASSOCIATED WITH AFLATOXIN PRODUCTION AND FACTORS AFFECTING PRODUCTION

*Aspergillus* species that produce aflatoxins are asexual fungi belonging to section *Flavis*. The species of this section include *A. flavus*, *A. parasiticus*, *A. nomius*, *A. oryzae*, *A. sojae* and *A. tamarii*. However, the fungi that exhibit aflatoxin production are mainly *A. flavus*, *A. parasiticus* and *A. nomius* (Bayman and Cotty, 1993). The aflatoxins produced by *A. flavus* and *A. parasiticus* are by far the most widely studied toxins. In their studies of the molecular aspects of aflatoxin biosynthesis by *A. tamarii*, *A. flavus* and *A. parasiticus*, Klich et al. (2000) found strong similarity among the biosynthesis pathway genes of these three species.

The ability to synthesize aflatoxins is strain-dependent. Baird et al. (2006) used nucleic acid profiling to analyze *A. flavus* isolates for aflatoxin production, in an attempt to differentiate aflatoxigenic from non-aflatoxigenic isolates. Synthesis may occur in both fungal hyphae and conidia. Maximum levels of aflatoxins are achieved when the fungal mycelium, or 'biomass' reaches an optimal level, above which the production rapidly declines, along with hyphal autolysis (Varma and Verma, 1987). Aflatoxins can also be synthesized in sclerotia produced by various isolates of *A. flavus* and *A. parasiticus* (Wicklow and Cole, 1982), in which it has been interpreted as a means of chemical defense of the fungus against potential invaders; it may be related to the importance of sclerotia in fungal survival.

The composition of the culture medium is an important factor in aflatoxin production. Trace elements, such as iron, copper and cadmium, were found to decrease aflatoxin production, whereas cobalt and zinc stimulated its production (Tiwari et al., 1986). Culture age markedly affected aflatoxin synthesis by *A. parasiticus*: the greatest amounts of aflatoxin were synthesized by young mycelia (4-days old) whereas aging mycelia (14-days old) mainly caused toxin degradation (Huynt and Lloyd, 1984).

The optimal temperature for fungal growth and aflatoxin production by the two high-temperature species, *A. flavus* and *A. parasiticus*, is 35°C with 0.95  $w_a$  and 33°C with 0.99  $w_a$ , respectively. Neither species produces aflatoxins when grown below 7.5°C or above 40°C. It was emphasized that the range for aflatoxin production was narrower than that for growth (Hill et al., 1985).

The composition of the atmospheric gases around the toxigenic fungus is another factor affecting toxin production. Controlled atmospheres or modified atmospheres produced within suitable packaging films were found capable of suppressing aflatoxin production by *A. flavus* (Clevström et al., 1983; Ellis et al., 1993).

### 6.3.3. AFLATOXIN OCCURRENCE IN FRUITS

The natural occurrence of aflatoxins in several agricultural commodities, such as cereals, cotton and groundnuts, may be a worldwide problem, since the majority of the crops involved are traded in international commerce (Smith, 1997). Numerous studies have been focused on the susceptibility of groundnuts and corn to aflatoxigenic fungi and aflatoxin contamination (Ioannou-Kakouri et al., 2001). During the last decades various fruits have been added to the list of products exposed to contamination by aflatoxins. Aflatoxins have often been found in fruits grown under tropical and subtropical climates, where the typical environmental conditions support growth of mycotoxigenic molds, and the subsequent aflatoxin production.

The effect of carbohydrates in the fruit on toxin production was shown by Le Bars (1990), who found that the ripe fig fruit served as a suitable medium for aflatoxin production, probably because of its high content of carbohydrates. Such conditions were suggested to be more favorable for toxin formation than for fungal growth. Morton et al. (1979) indicated that other carbohydrate-rich fruits are also good substrates for aflatoxin production, but emphasized the advantage of dried figs over dried apricots, pineapples and raisins.

One of the features that might affect the pathogenicity of *A. flavus* and *A. parasiticus* and their aflatoxigenic potential is their ability to produce ethylene during early growth stages (Sharma et al., 1985). Ethylene, which is frequently called 'the fruit-ripening hormone', is a natural biologically active compound which was found to inhibit aflatoxin production by *A. parasiticus* at 0.1–150 ppm. However, several factors, such as the medium, the temperature and the presence of CO<sub>2</sub> or the ethylene-inhibiting compound

1-methylcyclopropane, may alter the effects of ethylene. It was suggested that ethylene modulates toxin biosynthesis by the fungus via an ethylene sensor, and that the inhibitory effect of ethylene occurs at the level of transcription (Roze et al., 2004).

Natural contamination by aflatoxins has been reported in several fresh and dried fruits including figs, dates, citrus fruits, raisins and tree nuts, whereas aflatoxigenic potential of dried apricots and pineapples has also been recorded (Morton et al., 1979).

#### 6.3.3.1. Aflatoxins in figs

Figs are susceptible to infection by *A. flavus* conidia, which naturally contaminate the fruit surface. However, sensitivity of figs to infection has been related to fruit age: young green figs are resistant to *A. flavus* invasion, but as the fruits ripen and become softer they lose their resistance (Buchanan et al., 1975). The susceptibility of figs to *A. flavus* has been associated with the ability of the pathogen to penetrate the internal cavity of the fruit. It also was suggested that when fruit juice is present on the fruit surface it stimulates germination, facilitates penetration and advances fungal colonization (Drusch and Ragab, 2003). Studies by Bourda et al. (1994) in northern Africa showed that *A. flavus* grew best after the figs became ripe but before they were dried completely.

Studying the distribution of aflatoxin within naturally contaminated dried figs, Steiner et al. (1988) pointed out that whereas only a small number of figs were contaminated, the levels of contamination in individual fruits were very high. Since a bright greenish-yellow fluorescence under UV light (365 nm) correlated qualitatively with the occurrence of aflatoxin contamination in the fig, Steiner et al. (1988) succeeded in removing most of the highly contaminated figs from the sample and thereby lowered the level of aflatoxin B<sub>1</sub> from 22.6 to 0.3 ppb. In a later study carried out on naturally infected figs by Doster and Michailides (1998) in California the greenish-yellow fluorescence of dried figs under UV light was associated with decay caused by the two aflatoxigenic species, *A. flavus* and *A. parasiticus*, and the two non-aflatoxigenic species, *A. tamarii* and *A. alliaceus*. Although many of the non-fluorescent figs had small fungal colonies, that was not the rule. It was suggested that it might be possible to exploit the occurrence of fluorescence in infected figs to help the removal of aflatoxin-contaminated fruits in California only in particular situations, such as in cases where the contamination is superficial.

Aflatoxins B<sub>1</sub>, B<sub>2</sub> and G<sub>1</sub> were produced only in low-grade fig samples collected while drying; the highest level was recorded for aflatoxin B<sub>1</sub> (Boyacioglu and Gonul, 1990). Some of the samples contained all the three aflatoxins, the average contents being 112.3, 50.6 and 61.4 ng/g for B<sub>1</sub>, B<sub>2</sub> and G<sub>1</sub>, respectively.

Both high incidence and high levels of aflatoxin contamination were recorded in dried figs from Turkey (Gelosa, 1990) and from both dried figs

and fig pastes imported from Turkey to the UK (Sharman et al., 1991). Aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> were found by Özey and Alperden (1991) in 29 percent of the dried fig samples collected from orchards in Turkey. Natural contamination occurred, however, only under high temperature and high humidity conditions (Özey et al., 1995). In California aflatoxins at levels above 100 µg/kg were detected in 83 percent of the figs from orchards infected by *A. parasiticus*, but in only 32 percent of those from orchards infected by *A. flavus* (Doster et al., 1996). All the figs infected with *A. parasiticus* contained both aflatoxins, B and G, whereas those infected with *A. flavus* did not contain any G toxins. However, particularly high levels of aflatoxin were recorded in individual figs naturally infected by *A. flavus*, with levels of 1800 and 9600 µg/kg, and in those infected by *A. parasiticus*, with levels of 17 900 and 77 200 µg/kg (Doster et al., 1996). Some samples of local figs in Cyprus were found to be contaminated with aflatoxins, but the concentrations (up to 5 µg/kg) were below the maximum permitted levels (10 µg/kg) (Ioannou-Kakouri et al., 2001).

### 6.3.3.2. Aflatoxins in dates

Dates, which are grown under high humidity and moderate temperatures, are one of the fruits that suffer from contamination by aflatoxins. Low levels of aflatoxin B<sub>1</sub> were reported by Herry and Lemetayer (1992) in 2 out of 28 date samples in Greater Glasgow. Studies in Egypt on local and imported dates revealed the presence of *A. flavus* strains capable of producing aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> (Emam et al., 1994). Dates imported to Europe or to the Middle East and North Africa were found to be contaminated with the two aflatoxigenic species, *A. flavus* and *A. parasiticus*, along with potentially pathogenic bacteria, lactic acid bacteria and yeasts (Aidoo et al., 1996). The stimulatory effect of date extracts on aflatoxin production was emphasized by Ahmed and Robinson (1999).

In their study on aflatoxins in fresh dates, Shenasi et al. (2002) found that *A. flavus* was present in more than 50 percent of the date samples. None of the 10 date varieties examined showed the presence of aflatoxin at any stage of maturation of the fresh samples, indicating that all the dates were initially free of aflatoxins. However, after storage under elevated humidity and at 30°C significant levels of aflatoxin B<sub>1</sub> were detected in three date varieties, at concentrations ranging from 35 to 11 610 µg/kg. In contrast to dried figs, mycotoxins do not appear to be naturally associated with dried date fruits (Shenasi et al., 2002).

### 6.3.3.3. Aflatoxins in citrus fruits

Infection of orange peel by *A. flavus* and the subsequent aflatoxin production can take place following previous deterioration of the peel because of other microorganisms (Varma and Verma, 1987).

In studying the fate of aflatoxins in oranges inoculated with *A. parasiticus* during processing, Ragab et al. (1999) found that up to 69 percent of the aflatoxins remained in the peel, up to 13 percent remained in the pulp and no more than 35 percent appeared in the clear juice.

In a recent study, Bamba and Sumbali (2005) reported on the formation of aflatoxin B<sub>1</sub> by *A. flavus* isolates that infected sour limes in northern India: 60 percent of the pathogenic *A. flavus* strains were found to be aflatoxin producers, with toxin levels ranging from 141.3 to 811.7 µg/kg.

#### 6.3.3.4. Aflatoxins in raisins

Evaluating the incidence of mycotoxigenic fungi in raisins, Giridhar and Reddy (2001) found a total of 29 fungal species, of which *A. flavus* was the most commonly recorded, followed by *A. niger*, *A. fumigatus* and *Penicillium* species. Aflatoxin B<sub>1</sub> and gliotoxin were produced in raisins by *A. flavus* and *A. fumigatus*, respectively (Youssef et al., 2000).

#### 6.3.3.5. Aflatoxins in tree nuts and olive fruits

Among the various *Aspergillus* species isolated from pistachio nuts in commercial orchards in California, *A. niger* was the only one that occurred frequently (in 30 percent of the nuts). The two aflatoxigenic fungi, *A. flavus* and *A. parasiticus*, were found at the early splits occurring in nuts in most orchards, and aflatoxins were detected in more than half of the orchards examined (Doster and Michailides, 1994).

A limited survey for the presence of aflatoxins in tree nuts from local markets and supermarkets in Valencia was carried out by Blesa et al. (2004). The samples containing aflatoxins were hazelnuts, with aflatoxin B<sub>1</sub> and G<sub>1</sub> levels up to 0.42 and 0.52 µg/kg, respectively, and pinhole with aflatoxin G<sub>1</sub> at 0.30 µg/kg. Mean levels of 3.6 and 0.15 µg/kg of aflatoxin were detected in aflatoxin B<sub>1</sub>-contaminated pistachios and almonds, respectively (Herry and Lemetayer, 1992). Such values are below the legislated maximum residue levels for the European Union. The four aflatoxins, B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> were found to contaminate pistachios of various geographic origins (Barbagallo and Russo, 1999). High levels of aflatoxin were also reported in pistachios available in Qatar with levels up to 289 and up to 81.6 µg/kg in studies carried out by Abdulkadar et al. (2000, 2004).

*Aspergillus flavus* strains isolated from Moroccan olive fruits were found to produce aflatoxin B<sub>1</sub> at concentrations between 48 to 95 µg/kg when grown on starch-based media (Roussos et al., 2006).

#### 6.3.3.6. Occurrence of aflatoxins in fruits – A summarizing table

The occurrence of aflatoxins in fresh and dried fruits, as well as in tree nuts, is summarized in Table 6.2.

TABLE 6.2 Aflatoxins in fruits, dried fruits and tree nuts.

Toxin	Fruits	Maximum (mean) toxin level (µg/kg)	References
<b>Fresh fruits</b>			
Aflatoxin B <sub>1</sub>	Oranges	52	Ragab et al., 1999
Aflatoxin B <sub>1</sub> , B <sub>2</sub> , G <sub>1</sub> , G <sub>2</sub>	Oranges	120	Ragab et al., 1999
Aflatoxin B <sub>1</sub>	Sour limes	811.7	Bamba and Sumbali, 2005
Aflatoxin B <sub>1</sub> , B <sub>2</sub> , G <sub>1</sub> , G <sub>2</sub>	Apples	350	Hasan, 2000
Aflatoxin B <sub>1</sub>	Dates	11610 <sup>a</sup>	Shenasi et al., 2002
<b>Dried fruits</b>			
Aflatoxin B <sub>1</sub> , B <sub>2</sub>	Figs	30	Steiner et al., 1988
Aflatoxins	Figs	241	Roy, 1990
Aflatoxins	Figs	337	Gelosa, 1990
Aflatoxin B <sub>1</sub>	Figs	320 <sup>b</sup>	Boyacioglu and Gonul, 1990
Aflatoxin B <sub>2</sub>	Figs	71.8 <sup>b</sup>	Boyacioglu and Gonul, 1990
Aflatoxin G <sub>1</sub>	Figs	97.5 <sup>b</sup>	Boyacioglu and Gonul, 1990
Aflatoxin B <sub>1</sub>	Figs	63.0	Özay and Alperden, 1991
Aflatoxin B <sub>2</sub>	Figs	37.7	Özay and Alperden, 1991
Aflatoxin G <sub>1</sub>	Figs	78.3	Özay and Alperden, 1991
Aflatoxin G <sub>2</sub>	Figs	15.0	Özay and Alperden, 1991
Aflatoxins	Figs	96	Sharman et al., 1991
Aflatoxins	Fig paste	165	Sharman et al., 1991
Aflatoxins	Figs	(1.9)	Herry and Lemetayer, 1992
Aflatoxins	Figs	5.0	Ioannou-Kakouri et al., 2001
Aflatoxins	Figs	4.0	Senyuva et al., 2007
Aflatoxin B <sub>1</sub>	Raisins	300	Youssef et al., 2000
Aflatoxin	Raisins	ND	Abdulkadar et al., 2004
<b>Tree nuts</b>			
Aflatoxin B <sub>2</sub> , G <sub>2</sub>	Pistachios	45.07	Barbagallo and Russo, 1999
Aflatoxin B <sub>1</sub> , B <sub>2</sub> , G <sub>1</sub> , G <sub>2</sub>	Pistachios	87 <sup>c</sup>	Barbagallo and Russo, 1999
Aflatoxin B <sub>1</sub> , B <sub>2</sub> , G <sub>1</sub> , G <sub>2</sub>	Pistachios	102 <sup>d</sup>	Barbagallo and Russo, 1999
Aflatoxin B <sub>1</sub>	Pistachios	289	Abdulkadar et al., 2000
Aflatoxin B <sub>1</sub>	Pistachios	81.6	Abdulkadar et al., 2004
Aflatoxin B <sub>1</sub>	Pistachios	(3.6)	Herry and Lemetayer, 1992
Aflatoxin B <sub>1</sub>	Almonds	(0.15)	Herry and Lemetayer, 1992
Aflatoxin B <sub>1</sub>	Hazelnuts	0.42	Blesa et al., 2004
Aflatoxin G <sub>1</sub>	Hazelnuts	0.52	Blesa et al., 2004

<sup>a</sup> In storage under elevated humidity at 30°C, at late stage of maturity.

<sup>b</sup> Collected from contaminated samples at the drying stage.

<sup>c</sup> Samples from Greece.

<sup>d</sup> Samples from Turkey.

ND – not detected.

## 6.4. OCHRATOXINS

### 6.4.1. OCHRATOXIN A (OTA) AND ITS TOXIC EFFECTS

Ochratoxins are secondary metabolites produced by various *Aspergillus* and *Penicillium* species that contaminate a wide range of foods, including a wide

variety of fruits and vegetables. They are a group of isocoumarin derivatives, of which OTA ( $C_{20}H_{18}O_6NCl$ ) is the most abundantly produced and the most toxic compound (Abramson, 1997).

By using a preparation of monoclonal antibodies against OTA, Varga et al. (1996) found that OTA can be produced by strains of *A. ochraceus*, *A. alliaceus*, *A. sclerotiorum*, *A. sulphureus*, *A. albertensis*, *A. auricomus* and *A. wentii*.

The classic human dietary sources of OTA have been cereals, legumes, coffee-beans, cocoa-beans, grapes, dried vine fruits, nuts and spices (Frisvad, 1995), but they may also include meat products, as a result of contaminated animal feed (Aish et al., 2004). During the last decade the importance of wines and grape juices as sources of OTA in human dietary has been increasingly recognized following worldwide surveys on the occurrence of OTA in these products (Battilani and Pietri, 2004; Battilani et al., 2006).

OTA is the mycotoxin most frequently found in the blood of people exposed to mycotoxins in their food (Sangare-Tigori et al., 2006). Following its absorption, this mycotoxin binds to the human serum albumin (Il'ichev et al., 2002). Its presence has been recorded in breast milk (Creppy, 1999) and in both mothers' milk and infants' sera (Hassan et al., 2006).

OTA is regarded as a major concern of livestock producers, and is the only ochratoxin that plays a role as an environmental toxin (Logrieco et al., 2003), although the production of ochratoxin B ( $C_{20}H_{19}O_6N$ ) by several *Aspergillus* species has been reported (Varga et al., 1996). It is toxic to various animals, primarily through its action as a nephrotoxin, and is among the strongest carcinogenic compounds that affect rat and mice, in which the kidney is the principal site and the liver the major secondary site of tumor formation (Aish et al., 2004). It also has been considered to be one of the main mycotoxin hazards to human health, as a factor in the human kidney disease known as Balkan Endemic Nephropathy, and in the development of urinary tract tumors (Creppy, 1999). In 1993 the International Agency for Research of Cancer (IARC, 1993) classified OTA as a possible human carcinogen (included in Group 2B).

Since OTA may contaminate wines naturally (Battilani and Pietri, 2004), Bertelli et al. (2005) studied the effect of wine and ethanol on the nephrotoxicity of OTA; they found that the red wine, but not ethanol, protected against the nephrotoxicity of OTA by limiting its oxidative damage.

OTA may also exhibit teratogenic, genotoxic and mutagenic effects, and also has been regarded as an immunosuppressive agent (Petzinger and Weidenbach, 2002). Its immunotoxic activity, as expressed in several vital immunity-related organs, has recently been discussed (Al-Anati and Petzinger, 2006).

The biochemical effect of OTA results primarily from its structural similarity to the essential amino acid, phenylalanine. The principal effect appears to be the inhibition of protein synthesis, although inhibition of RNA and DNA synthesis has also been implicated in its mechanism of action (Aish et al., 2004). Although exposure to the toxin is usually due to ingestion of contaminated food and feed, airborne invasion may also occur in rare cases (Richard et al., 1999).



6.4.2. OCHRATOXIN A IN FRUITS AND ITS SOURCES

Contamination of fruits and their products with a mycotoxin known to have carcinogenic, teratogenic and immunosuppressive properties is of major concern for human health.

6.4.2.1. Ochratoxin A in fruits with an emphasis on figs

Although the majority of recent studies on OTA in fruits have been focused on its presence in grapes and grape-based products, studies also have addressed OTA contamination of other fruits. Engelhardt et al. (1999) studied mycotoxin contamination in several fruits, after the removal of rotten tissue, and found OTA in cherries at levels up to 27.1 µg/kg, and lower levels in tomatoes, strawberries, apples and peaches (Table 6.3). These findings indicate the potential hazard in fruits intended for consumption, even after the removal of rotten parts.

*Aspergillus niger* strains isolated from olive fruits were capable of producing OTA when grown in vitro on a starch-based medium (Roussos et al., 2006).

Special attention has been drawn to OTA accumulation in fresh and dried figs. Doster et al. (1996) and Bayman et al. (2002) studied OTA production in figs grown in California. Commercially grown figs that were naturally infected with *A. alliaceous*, *A. melleus*, *A. ochraceus* and *A. sclerotiorum* (all belonging to the *Aspergillus* Section *Circumdati*) had OTA contents ranging from zero to 9600 µg/kg (Doster et al., 1996) (Table 6.3); about 40 percent of the fruits contained more than a trace amount of ochratoxin. Bayman et al. (2002) found that *A. ochraceus* and *A. melleus* were the most common species, followed by *A. alliaceous*, *A. elegans* and *A. sclerotiorum*. However, examination of 50 000

TABLE 6.3 Ochratoxin A in fresh and dried fruits (excluding grapes).

Fruits	Maximum (mean) toxin level (µg/kg)	References
<b>Fresh fruits</b>		
California figs	9600 <sup>a</sup>	Doster et al., 1996
Cherries, Germany	27.1	Engelhardt et al., 1999
Tomatoes, Germany	1.44	Engelhardt et al., 1999
Strawberries, Germany	1.44	Engelhardt et al., 1999
Apples, Germany	0.41	Engelhardt et al., 1999
<b>Dried fruits</b>		
Dried figs, Turkey	337	Gelosa, 1990
Dried figs, Turkey	8.3	Özay and Alperden, 1991
Dried figs, Turkey	26.3 <sup>b</sup>	Senyuva et al., 2005
Dried figs, Egypt	120	Zohri and Ardel-Gawad, 1993
Dried apricots, Egypt	110	Zohri and Ardel-Gawad, 1993
Dried plums, Egypt	280	Zohri and Ardel-Gawad, 1993

<sup>a</sup>Co-occurrence with aflatoxin B<sub>1</sub>.

<sup>b</sup>Only 40% of the figs contained more than a trace amount of OTA.

individual samples of figs with visible fungal colonies showed that the common aspergilli, *A. ochraceus* and *A. melleus*, did not produce detectable levels of OTA (i.e., above 0.01 µg/ml) when grown in vitro in liquid media. On the other hand, all the *A. alliaceus* isolates produced OTA at concentrations up to 30 µg/ml. Three results have thus clarified that the production of OTA in figs was connected with the presence of *A. alliaceus*: (a) none of the isolates of *A. ochraceus* or *A. melleus* (except for one strain) produced OTA at detectable levels; (b) all the isolates of *A. alliaceus* produced OTA in culture, some at high levels; and (c) the ochratoxin content of figs was correlated with the presence of *A. alliaceus* but not with aspergilli of the *A. ochraceus* group or with *Penicillium* species (Bayman et al., 2002).

Zohri and Ardel-Gawad (1993) studied the OTA contents in dried figs, apricots and plums in Egypt and found up to 280 µg/kg in dried plums (Table 6.3). In Turkey, where dried figs are economically important, Özay and Alperden (1991) analyzed 103 fig samples and found only 3 percent incidence of OTA, with concentrations of 5.2–8.3 µg/kg (Table 6.3). In a subsequent survey no OTA was detected in any of the 32 samples of dried figs examined (Özay et al. 1995); it was found that the application of solar drying to the figs contributed to the reduction of their contamination.

The presence of OTA and its co-occurrence with aflatoxin B<sub>1</sub> in dried figs were recorded in Turkey by Senyuva et al. (2005). The procedure employed for OTA detection included an alkaline extraction which gave consistently higher toxin recovery than the conventionally employed acid extraction. This sensitive methodology for OTA detection showed this mycotoxin to be more widespread than previously supposed. Quite similar results were recorded in 2003 and 2004: in the 2004 survey, 4 out of 41 fig samples contained only OTA, and two samples contained both OTA and aflatoxin B<sub>1</sub>, with a maximum OTA concentration of 26.3 ng/g (Table 6.3). Although OTA and aflatoxin B<sub>1</sub> were determined in separate laboratories, the simultaneous analysis of the two mycotoxins in some instances should be considered (Senyuva et al., 2005).

#### 6.4.2.2. Ochratoxin A in grapes – *Aspergillus carbonarius* the key producer

An increasing number of studies has recently been focused on OTA accumulation in grapes, dried vine fruits, wines and juices, and on the fungi responsible for its production. It generally has been stated that *Penicillium verrucosum* is the producer of OTA in temperate climates, whereas several *Aspergillus* species are the mycotoxin producers in subtropical and tropical climates (Abarca et al., 1994; Varga et al., 1996). Until 1996 *A. ochraceus* was believed to be the only *Aspergillus* species that produced ochratoxin in grapes, although the ability of *A. niger* var. *niger* to produce OTA had been reported (Abarca et al., 1994). Following the finding of significant levels of OTA by Zimmerli and Dick (1996) in grape juices and wines, the two black-spored *Aspergillus* species, *A. carbonarius* and *A. niger*, were regarded as the source of ochratoxin production.

The relevance of the black aspergilli, and particularly of *A. carbonarius*, to OTA production in grapes was pointed out in several countries and was reviewed

by Battilani and Pietri (2004). Studies by da Rocha et al. (2002) in Argentina indicated that although *A. ochraceus* was isolated at a very low incidence, 25 percent of the aspergilli belonging to the *A. niger* aggregate were ochratoxigenic. The dominance of the black aspergilli (*A. niger* aggregate) on several grape varieties was similarly recorded in Argentina by Magnoli et al. (2003) and Chulze et al. (2006), who found that about 41 percent of this population were OTA producers, with levels in the culture medium ranging from 2 to 24.5 ng/ml.

Studies by Sage et al. (2002) on OTA in grapes from southern France indicated that although 6 out of 11 grape samples were contaminated by potentially ochratoxigenic strains, *A. carbonarius* was the only species capable of producing OTA in culture. The levels recorded ranged from 0.5 to 87.5 µg/g. Of the rich aspergilli population recorded by Sage et al. (2004) in 60 French grape samples, only *A. carbonarius* strains were ochratoxigenic, producing ochratoxin at levels up to 1.9 µg/g.

Of the total *Aspergillus* species isolated from 10 vineyards with differing climatic conditions in France, the black aspergilli were by far the most common pathogens (99 percent) (Bejaoui et al., 2006). They were represented by three populations: *A. carbonarius*, *A. japonicus* and *A. niger* aggregate, of which *A. japonicus* was in the minority. *A. carbonarius* was the main OTA producer (up to 37.5 µg/g) and the harvest time was the critical period influencing OTA contamination.

In a study in vineyards in Italy, Battilani et al. (2003) found that whereas aspergilli belonging to section *Circumdati* (which includes *A. ochraceus*) were occasionally isolated, all the aspergilli belonging to section *Nigri* were identified as *A. carbonarius*. A study by Pollastro et al. (2006) in southern Italy showed that the frequency of occurrence of the predominant aspergilli on grapes diminished in the following order: *A. niger* > *A. carbonarius* > *A. aculeatus* > *A. wentii*. Of these, however, *A. carbonarius* was the main OTA producer. In studying the microflora of Portuguese wine grapes, Serra et al. (2003) indicated that most of the *A. carbonarius* strains (97 percent) and 4 percent of the *A. niger* aggregate strains were ochratoxin producers. It was later found that although *A. niger* aggregate was identified in 5.4 percent of the species collected, while *A. carbonarius* was found in only 0.6 percent of the species, OTA was detected in all the cultures of *A. carbonarius* and in only 4 percent of the *A. niger* aggregate strains, suggesting that *A. carbonarius* is most likely to occur in vineyards with Mediterranean climates (Serra et al., 2005). No ochratoxigenic aspergilli were found by Abrunhosa et al. (2001) in their earlier survey in the colder climate of northern Portugal.

In testing 386 isolates of *Aspergillus* section *Nigri* and 10 of *Aspergillus* section *Circumdati* from Spanish grapes, Bellí et al. (2004) found that isolates of section *Circumdati* produced greater amounts of OTA in vitro than those of section *Nigri* (mean levels in substrate of 10.76 and 1.42 µg/g, respectively). However, aspergilli of section *Nigri* were suggested to be responsible for the ochratoxin level in wines and musts (unfermented or incompletely fermented grape juice) since they were much more widespread in grapes. The relevance of section *Nigri* to OTA production was further confirmed by Bellí et al.

(2005), who found that of the total black aspergilli isolated from 40 vineyards in Spain, *A. carbonarius* formed the highest percentage of ochratoxin-positive strains and produced the highest levels of the toxin (2.5–25 µg/g).

Of the black aspergilli predominating in Spanish wine grapes, all (Bau et al., 2005) or most (Bellí et al., 2006b) of the *A. carbonarius* isolates were capable of producing OTA, particularly in their late stages of development, whereas none of the isolates of *A. niger* aggregate and *A. japonicus* var. *aculeatus* produced it.

Field sampling of two wine cultivars from Israel showed that 161 out of 2114 isolates were OTA producers, and that the biserial *A. carbonarius* was the most consistent toxin producer (35 percent), whereas only 3.1 percent of the isolates of *A. niger* aggregate were ochratoxigenic, and no OTA-producing uniseriate isolates were found (Guzev et al., 2006).

*A. carbonarius* was similarly found to be the primary OTA-producing species in grapes in Australia: *A. niger*, which was isolated more frequently from vineyards was only a poor toxin producer (Leong et al., 2006, 2007).

In studying the mycoflora of healthy (without visible symptoms of rot) wine grapes, Serra et al. (2006a) isolated three *Aspergillus* species from healthy grapes that were still in the vineyards: *A. carbonarius*, *A. niger* aggregate and *A. ochraceus*. Of these, only *A. carbonarius* and *A. ochraceus* were capable of ochratoxin production at detectable levels, and only *A. carbonarius* was found to produce the toxin. The presence of OTA in healthy berries confirmed the ability of ochratoxigenic fungi to infect berries in the vineyard.

A study on ochratoxigenic fungi in grapes intended for liqueur wine preparation in Barcelona by Gómez et al. (2006) showed that *A. carbonarius* was the major source of OTA in these wines as well. Since the production of liqueurs requires overripening of the grapes on the vine, or extended post-harvest exposure of the grapes in the sun, the grapes were examined during their developmental stages. Increases in *Aspergillus* spp. were recorded as the berries matured, and black aspergilli (*A. niger* aggregate and *A. carbonarius*) were the predominant fungi. At harvest and after overripening, the percentage of *A. carbonarius* exceeded that of *A. niger* aggregate, and above 98 percent of the *A. carbonarius* isolates were ochratoxin producers.

Most of the studies of OTA occurrence in grapes grown in the Mediterranean region have clearly emphasized that, although the contribution of *A. carbonarius* to ochratoxin accumulation in grapes has only recently been recognized, this species is now regarded as the major source of ochratoxin in grapes and their products. Since other aspergilli of section Nigri are frequently found on grapes, along with *A. carbonarius*, a semi-selective medium based on Malt Extract Agar amended with antibiotics and fungicides has been developed for identification and isolation of *A. carbonarius* (Polastro et al., 2006).

#### 6.4.2.3. Contributions of *Aspergillus tubingensis* and *A. niger* to ochratoxin A production in grapes

In a study carried out by Medina et al. (2005) in five grape varieties from Spain, two *Aspergillus* species section Nigri were found to be ochratoxigenic: *A. carbonarius*

and *A. tubingensis*, of which 74.2 and 14.3 percent, respectively, were OTA producers. The levels of ochratoxin produced by the two species ranged from 1.2 to 3530 and from 46.4 to 111.5 ng/ml, respectively.

The capabilities of *Aspergillus* species section Nigri other than *A. carbonarius* to produce OTA in grapes were investigated in 77 black aspergilli isolated by Perrone et al. (2006) in 16 vineyards in Italy. By using amplified fragment length polymorphisms (Alps) and genomic DNA sequences four main groups that shared an AFLP similarity of <25 percent were distinguished: (1) 22 of 23 *A. carbonarius* strains that produced OTA at 6–7500 µg/l; (2) five of 20 *A. tubingensis* strains that produced OTA at 4–130 µg/l; (3) three of 15 *A. niger* strains that produced it at 250–360 µg/l; and (4) all the 19 uniseriate aspergilli tested that did not produce OTA at detectable levels, i.e., above 4 µg/l. This identification clarified that *A. tubingensis* is capable of producing OTA and that, together with *A. carbonarius* and *A. niger*, it may be among the sources of OTA in wines in Italy (Perrone et al., 2006).

#### 6.4.2.4. Factors affecting ochratoxin A production in grapes

Studies in vitro indicated that most *A. carbonarius* strains grew optimally at 30–35°C, with little growth at <15°C (Mitchell et al., 2004; Leong et al., 2006). Marin et al. (2006) indicated that OTA was rarely detected at 35°C although growth was maximal at this temperature. Mitchell et al. (2004) studied the effect of water availability on *A. carbonarius* growth, and found that the optimum  $a_w$  for growth varied from 0.93 to 0.987, with the widest  $a_w$  tolerance at 25–30°C.

Grapes were found to be susceptible to ochratoxigenic aspergilli early in their developmental stages, starting from about one month or more after setting, when *Aspergillus* species were found on grapes in a few vineyards (Battilani et al., 2003). At early veraison and ripening aspergilli were present in all the vineyards tested and the highest levels of OTA were recorded. The rate of fungal growth on grapes depended on the species and isolates involved: *A. carbonarius* was very invasive and penetrated even berries without skin damage, whereas another contaminating species, *A. fumigatus*, colonized the pulp only in damaged berries. The amount of OTA produced was thus influenced by both the fungal isolate and the state of the berries.

Differences in ochratoxin content in Italian berries, of the same cultivar and managed similarly, were related to the geographical area. The temperature, rainfall and relative humidity were suggested to be the main factors that distinguished the different geographic areas (Battilani et al., 2003). Higher ochratoxin concentrations were generally recorded in wines made from grapes grown in the southern than in the northern regions of Italy. This is in good agreement with the findings of higher OTA contents in the southern than in the northern European regions reported by Zimmerli and Dick (1996) and by Otteneder and Majerus (2000). The importance of the geographic area is expressed in the fact that the Mediterranean basin is particularly affected. This region includes the southern areas of France and Italy, Greece and certain

areas of Portugal and Spain (Varga and Kozakiewicz, 2006). Both climatic conditions and the year of sampling affected mycotoxin contamination of grapes and their derived products (Lopez de Cerain et al., 2002; Battilani et al., 2003; Stefanaki et al., 2003; Meyvaci et al., 2005).

A positive correlation was found between the number of black aspergilli isolated from grapes and the temperature in the field: the most contaminated grapes were those isolated in the warmest year and from the warmest regions (Battilani et al., 2003, 2006). This indicated that the preharvest period plays an important role in determining ochratoxin contents in grapes.

Bellí et al. (2006a) studied the effects of temperature regimes that simulated preharvest day and night conditions in the field on *A. carbonarius* growth and OTA production. A 12-hour photoperiod stimulated fungal growth and thereby enhanced OTA accumulation, although it had no direct effect on OTA production. A significant positive correlation was found between the black *Aspergillus* infection of wine grapes and the temperature in the month preceding the fruit sampling, whereas no significant correlation was found with the relative humidity or rainfall.

The condition of the grapes is particularly important with regard to the expected OTA contamination. Berries injured by insects or by excessive irrigation or rain are liable to contain more OTA than healthy berries (Varga and Kozakiewicz, 2006). Leong et al. (2006, 2007) emphasized that damage to berries is a primary factor that affects bunch rots and the subsequent production of OTA in grapes. In the absence of damage *Aspergillus* spores may exist on berry surfaces without causing visible rot. The significance of damage to grape bunches at the period between veraison and harvest, which is the critical period for OTA production, may therefore result in significance reduction of *Aspergillus* rot and ochratoxin production (Leong et al., 2007).

OTA can already be produced at early maturation stages (Serra et al., 2006b). Its level in grapes was found to increase as the fruits matured (Lataste et al., 2004) or as the berries developed (Bau et al., 2005). The longer the grapes were on the vine, the more ochratoxigenic aspergilli appeared, reaching 100 percent infection at harvest (Bellí et al., 2004).

Another factor that may affect OTA production by *A. carbonarius* in grapes is the possible interaction with other fungi present on the maturing berries. Valero et al. (2006) studied the effects of several fungi isolated from grapes on OTA production, and found that *Alternaria alternata*, *Cladosporium herbarum*, *Penicillium decumbens*, *P. janthinellum* and *Trichoderma harzianum* suppressed mycotoxin production when grown in vitro at 30°C, whereas *Erotium amstelodami* and *Candida* spp. seemed to stimulate production. Such effects were not recorded at 20°C, the optimal temperature for OTA production.

Since acquisition of knowledge of the environmental conditions required for the successive stages of fungal development is the first step towards preventing mycotoxin production, Pardo et al. (2006) focused on the ecophysiology of two ochratoxigenic fungi, *A. ochraceus* and *P. verrucosum*. Predictive models for different stages of fungal development were developed, which enabled the prediction of the time before the occurrence of spoilage as a

function of the abiotic factors. The application of these studies in the management of grapes has been proposed as a means to help identifying the critical control points (CCPs) in the production, storage and distribution processes.

#### 6.4.2.5. Ochratoxin A in dried vine fruits

Dried vine fruits (raisins, sultanas, currants) are regarded as 'healthy foods' and are also ingredients in muesli, biscuits, cakes and other foods. Although above 50 percent of the dietary OTA intake in Europe was estimated to derive from cereals, dried vine fruits can also be an important dietary source of OTA for people consuming large amounts of these fruits, and particularly for children (Abarca et al., 2003).

Studying the presence of OTA in currants, sultanas and raisins purchased in the UK, MacDonald et al. (1999) detected the mycotoxin in 19, 17 and 17 samples, respectively, out of 20 samples of each dried fruit. The highest mean levels of OTA (up to 53.6 µg/kg) were recorded in currants. Studies of OTA production in dried fruits in Greece indicated that sultanas were generally less contaminated than currants, but great differences were recorded between different regions, and between different years in the same region. Thus, maximal levels in currants from the Peloponnese were 12.4 and 4.9 µg/kg in 1998 and 2000, respectively (Stefanaki et al., 2003).

The major contribution of *A. carbonarius* to OTA accumulation in dried fruits was confirmed in various countries. Although *A. niger* var. *niger* was detected in 98 percent of the samples whereas *A. carbonarius* was found in 58 percent of the samples, above 96 percent of *A. carbonarius* isolates and only 0.6 percent of those of *A. niger* var. *niger* were OTA producers (Abarca et al., 2003). High levels of OTA (up to 5200 µg/kg) were recorded in 83 percent of the *A. carbonarius* isolates from dried vine fruits from Argentina (Chulze et al., 2006).

The finding that in dried fruits strains of *A. carbonarius* were less common than those of *A. niger* aggregate, but more efficient OTA producers has arisen in several surveys (Abarca et al., 2003; Magnoli et al., 2004; Tjamos et al., 2004; Iamanaka et al., 2005; Romero et al., 2005). Some observations indicated that higher levels of OTA were recorded in black than in white sultanas (Magnoli et al., 2004; Iamanaka et al., 2005). The presence of other fungi on dried vine fruits was reported by Romero et al. (2005); they included *A. ochraceus* isolates, of which only a few were OTA producers, *A. flavus* isolates, of which only a few produced cyclopiazonic acid, and several *P. citrinum* isolates which were strong citrinin producers.

Iamanaka et al. (2005) analyzed various dried fruits sold in Brazil (including black and white sultanas, figs, dates, plums and apricots) for the presence of OTA, and found that the black sultanas and dried figs contained the highest levels of contamination, with 33 and 26.3 percent, respectively, of the samples containing OTA levels above 5 µg/kg, whereas no sample of the white sultanas, dates and plums exceeded this limit, and the apricot samples were all free of contamination. The highest contamination (33.9 µg/kg) occurred in one sample of black sultanas. *A. niger* was the most common species on the dried fruits,



TABLE 6.4 Ochratoxin A in dried vine fruits.

Fruit	Maximum (mean) toxin level (µg/kg)	References
Currants, UK	(53.6)	MacDonald et al., 1999
Sultanas, UK	(20)	MacDonald et al., 1999
Raisins, UK	(18.1)	MacDonald et al., 1999
Raisins, Egypt	250	Youssef et al., 2000
Dried figs, UK	>10	Min. Ag. Fish and Food, 2002
Currants, Peloponissos, Greece <sup>a</sup>	12.4	Stefanaki et al., 2003
Sultanas, Peloponissos, Greece <sup>a</sup>	3.6	Stefanaki et al., 2003
Sultanas, Crete, Greece <sup>a</sup>	13.2	Stefanaki et al., 2003
Currants, Canada	4.85	Lombaert et al., 2004
Raisins, Canada	26.1	Lombaert et al., 2004
Sultanas, Canada	26.0	Lombaert et al., 2004
Vine fruits (black) Argentina	14.0	Magnoli et al., 2004
Vine fruits (white), Argentina	7.5	Magnoli et al., 2004
Raisins, Qatar	1.2	Abdulkadar et al., 2004
Black sultanas, Brazil	33.9	Iamanaka et al., 2005
White sultanas, Brazil	5	Iamanaka et al., 2005
Dates and plums, Brazil	5	Iamanaka et al., 2005
Apricots, Brazil	ND <sup>b</sup>	Iamanaka et al., 2005
Figs, Brazil	30	Iamanaka et al., 2005
Raisins purchased in Hungary	6.2	Varga et al., 2006

<sup>a</sup>For the year 1998.

<sup>b</sup>ND – not detected.

but only 15 percent of its isolates were ochratoxigenic, whereas 87 and 60 percent of the isolates of the less common fungi, *A. ochraceus* and *A. carbonarius*, respectively, were ochratoxigenic.

The UK Ministry of Agriculture, Fisheries and Food (MAFF) studied 61 dried fruit samples including dates, plums, apricots and figs and found only one dried fig sample contaminated with OTA at more than 10 µg/kg (Ministry of Agriculture, Fisheries and Food, 2002) (Table 6.4).

The dominance of OTA-producing fungi in sun-dried grapes intended for special wine production was attributed to the ability of aspergilli belonging to section Nigri to grow in a wide range of temperatures, and their probable better adaptation to hot and humid environment (Valero et al., 2005). Studies on OTA production in drying currants suggested that the increase in mycotoxin contamination occurs during the 7- to 14-day drying process, and may be influenced by the prevailing weather conditions and the drying rates (Magan and Aldred, 2005).

6.4.2.6. Ochratoxin A in fruit juices

Low levels of OTA, slightly above the limit of detection, were found in Germany by Majerus et al. (2000) in blackcurrant, tomato and carrot juices, whereas apple and orange juices were free of ochratoxin A. Filali et al. (2001) analyzed 14 samples of various fruit juices (orange, mango, pineapple, clementine,



grapefruit and peach juices) from Morocco, and found that whereas the grapefruit juice contained OTA at 1160 ng/l, no ochratoxin was recorded in the other samples at levels above the quantification limit of 0.01 µg/l.

Most of the studies focused on OTA in grape juices. The presence of OTA in red grape juice was reported by Zimmerli and Dick (1996), and higher concentrations were recorded in red than in white grape juices by Majerus et al. (2000) and Ng et al. (2004). OTA was also found in French, Spanish and Tunisian musts (Sage et al., 2002; Belli et al., 2004; Fredj et al., 2007) as well as in pulp of frozen grapes in Brazil (Rosa et al., 2004). Only low concentrations were found in 2 out of 12 juice samples from Japan, with an average of 6 ng/l (Kawamura, 2005) (Table 6.5).

Although grape juice is generally regarded as a niche product on the market, the fact that the consumers are often children emphasizes the importance of OTA in the juice in relation to the total intake of the mycotoxin by children (Jørgensen, 2005).

TABLE 6.5 Ochratoxin A in fruit juices, musts, wines and vinegars.

Juices, musts and fruit pulp	Maximum (mean) toxin level (ng/l)	References
Red grape juice, Switzerland	311	Zimmerli and Dick, 1996
Tomato juice, Germany	32	Majerus et al., 2000
Blackcurrant juice, Germany	60	Majerus et al., 2000
Red grape juice, Germany	5300	Majerus et al., 2000
White grape juice, Germany	1300	Majerus et al., 2000
Grapefruit juice, Morocco	1160	Filali et al., 2001
Pulp of frozen grapes, Brazil	35.4	Rosa et al., 2004
Red grape juices, Brazil	100	Rosa et al., 2004
Red juice, Canada and USA	104	Ng et al., 2004
White juice, Canada and USA	71	Ng et al., 2004
Grape juices and drinks, Poland	64.7	Czerwiecki et al., 2005
Grape juices, Japan	(6)	Kawamura, 2005
French musts	461	Sage et al., 2002
Spanish musts	813	Belli et al., 2004
Tunisian musts	4300	Fredj et al., 2007
<b>Wines</b>		
Red wine, Switzerland	388	Zimmerli and Dick, 1996
Rosé wine, Switzerland	123	Zimmerli and Dick, 1996
White wine, Switzerland	178	Zimmerli and Dick, 1996
Red wine, Germany	7000	Majerus and Otteneder, 1996
Rosé wine, Germany	2400	Majerus and Otteneder, 1996
White wine, Germany	1200	Majerus and Otteneder, 1996
Red wine, France	270	Ospital et al., 1998
Rosé wine, France	110	Ospital et al., 1998
White wine, France	20	Ospital et al., 1998
Commercial red wine, Italy	7630	Visconti et al., 1999
Commercial rosé wine, Italy	1150	Visconti et al., 1999
Commercial white wine, Italy	970	Visconti et al., 1999
European red wine	603	Burdaspal and Legarda, 1999

TABLE 6.5—(Cont'd)

Juices, musts and fruit pulp	Maximum (mean) toxin level (ng/l)	References
European rosé wine	161	Burdaspal and Legarda, 1999
European white wine	267	Burdaspal and Legarda, 1999
Southern Italy red wine	3177	Pietri et al., 2001
Southern Italy dessert wine	3856	Pietri et al., 2001
Morocco red wine	3240	Filali et al., 2001
Morocco rosé wine	540	Filali et al., 2001
Morocco white wine	180	Filali et al., 2001
Mediterranean red wine	3400	Markaki et al., 2001
Spanish red wine	316	Lopez de Cerain et al., 2002
Spanish white wine	208	Lopez de Cerain et al., 2002
Northern Greece red wine	2690	Stefanaki et al., 2003
Northern Greece rosé wine	1160	Stefanaki et al., 2003
Northern Greece white wine	1720	Stefanaki et al., 2003
Dessert wines, Greece	2820	Stefanaki et al., 2003
South African red wine	380 <sup>a</sup>	Shephard et al., 2003
South African white wine	300 <sup>a</sup>	Shephard et al., 2003
Portuguese wine	2100	Ratola et al. 2004
Brazilian red wine	42.4	Rosa et al., 2004
Brazilian white wine	28.2	Rosa et al., 2004
Brazilian rose wine	35.4	Rosa et al., 2004
Argentina red wine	42.4	Rosa et al., 2004
Argentina white wine	ND <sup>b</sup>	Rosa et al., 2004
Chile red wine	70.7	Rosa et al., 2004
Canadian red wine	393	Ng et al., 2004
Canadian white wine	156	Ng et al., 2004
Red wine, Japan	(4)	Kawamura, 2005
Red wine from Italy	(47)	Kawamura, 2005
Red wine from France	(77)	Kawamura, 2005
Red wines, Poland	6710	Czerwiecki et al., 2005
<b>Vinegars</b>		
Wine vinegar	1900	Majerus et al., 2000
Balsamic vinegar	252	Markaki et al., 2001

<sup>a</sup>Bottled samples.  
<sup>b</sup>ND – not detected (<21 ng/l).

6.4.2.7. Ochratoxin A in wines

*Factors affecting ochratoxin A contamination in wine* In their study on OTA in European wines, Zimmerli and Dick (1996) suggested that the mycotoxin is produced after the grape harvest, but prior to the alcoholic fermentation. It was later found that the production of OTA may take place in the vineyard, where several factors affect fungal development and ochratoxin accumulation (Battilani et al., 2003; Battilani and Logrieco, 2006). Since OTA production in wines is primarily dependent on the presence of ochratoxigenic fungi in the berries, all the factors that favor fungal growth should be considered to affect mycotoxin production. These factors include: (1) climatic conditions and

latitude, which is associated with climatic conditions (Battilani et al., 2003; Blesa et al., 2006); (2) contact between the berries and relevant mycotoxigenic fungi; (3) wounds or injuries that allow fungus penetration into the fruit and predispose it to infection, and which may occur during harvest or as a result of extreme temperatures in the vineyard (Barkai-Golan, 2001); (4) grape cultivation practices, including the use of fungicidal treatments and phytosanitation (Lopez de Cerain et al., 2002; Blesa et al., 2006); and (5) the wine-making techniques, including the type of maceration and conditions of fermentation (Lopez de Cerain et al., 2002; Blesa et al., 2006).

Cabañes et al. (2002) suggested that, among the wine-making techniques, the methods used for the removal of water from the grapes, which involve high temperatures and exposure to strong sunlight, are relevant factors. However, *A. carbonarius* and other species of *Aspergillus* section *Nigri* are naturally protected from sunlight and UV light because of their black spores (Pitt and Hocking, 1997).

**Occurrence of ochratoxin A in wine** Particular attention has been drawn to the ochratoxin content in wines, because of the hazard that this mycotoxin may pose to human health worldwide. OTA in red, rosé and white table wines from Switzerland was first recorded by Zimmerli and Dick (1996) at maximum levels of 388, 123 and 178 ng/l, respectively. Majerus and Otteneder (1996) reported on higher maxima of OTA in wines from Germany, with levels up to 2400 ng/l in red wines. In Italy, high levels of OTA were reported by Visconti et al. (1999) in red and rosé wines but not in white wines, and by Pietri et al. (2001) in red wines from the southern regions. High levels were found in wines from Greece (Stefanaki et al., 2003), from the Mediterranean countries (Markaki et al., 2001), from Morocco (Filali et al., 2001), from Portugal (Ratola et al., 2004) and from the Polish market (Czerwiecki et al., 2005). In Spain, Burdaspal and Legarda (1999) recorded levels up to 603 ng/l in red wines, and lower levels in rosé and white wines (Table 6.5); particularly high contamination was recorded in dessert wines, with a mean level of 1048 ng/l. No OTA was detected in any of the Port Wine or Vinho Verde samples screened in Portugal (Festas et al., 2000). OTA was detected in 97.7% of wine samples in Hungary, with higher means for the red wines than for the white wines (Varga et al., 2005).

All the local South African wines tested by Shephard et al. (2003) contained detectable levels of OTA, with maxima of 380 and 330 ng/l for red and white bottled wine, respectively, which are below the suggested European Union limit of 500 ng/l (Table 6.5). Detectable levels of OTA were also recorded in wines sold in Canada (Ng et al., 2004), with up to 393 and 156 ng/ml in red and white wines, respectively. Lower levels were recorded by Rosa et al. (2004) in wines from Brazil and Argentina, and none of the wines analyzed by Pacin et al. (2005) in Argentina and Chile were contaminated. Kawamura (2005) examined OTA contamination in commercial wines in the Japanese market and found that 6 out of 20 samples of local red wine contained an average of 4 ng/l, all the five red wine samples from Italy and

the seven red wine samples from France contained average levels of 47 and 72 ng/l, respectively (Table 6.5); OTA was not detected in any of the red wine samples from the USA, Australia, Chile and South Africa. LanChi et al. (2005) detected OTA at up to 0.5 ppb in 50 percent of red wine samples surveyed in Taiwan.

The higher levels of OTA in red and rosé wines than in white wines have frequently been emphasized, and in many countries the OTA levels in wines have been reported to diminish in the order: red > rosé > white (Majerus et al., 2000; Ottener and Majerus, 2000; Filali et al., 2001; Markaki et al., 2001; Lopez de Cerain et al., 2002; Ng et al., 2004; Ratola et al., 2004; Rosa et al., 2004; Anli et al., 2005; Varga et al., 2005). These differences indicate that, in addition to the vineyard region, the wine color seems to be involved in toxin production (Table 6.5). According to Ottener and Majerus (2000) and Rosa et al. (2004), color gradient may be connected with the length of time the mash stands. Another hypothesis relates to the fact that white wines are clarified by the addition of clays, such as bentonite or zeolite, which might adsorb ochratoxin (Huwig et al., 2001). The differences between the ochratoxin contents of distinct types of wines could be related to differences in the wine-making processes (Serra et al. 2006a). No differences between red and white wines were recorded in South Africa (Standar and Steyn, 2002) and in Australia (Leong et al., 2006). Following the many studies on OTA contamination of wines, it has been suggested that next to cereals, wine is the most important source of OTA in our daily diet (Battilani et al., 2006).

The possible year-to-year variation in the OTA content of wine from a given area, as was found in wine from Greece (Stefanaki et al., 2003), raised the question of which data should be considered representative, particularly for the estimation of the intake among a given human population (Jørgensen, 2005).

Low levels of ochratoxin (up to 250 ng/l) were found in vinegar, especially balsamic vinegar (Markaki et al., 2001). Majerus et al. (2000) reported on the presence of OTA in 50 percent of the wine vinegar samples analyzed; its concentration ranged from 10 to 1900 ng/l. Markaki et al. (2001) analyzed 15 balsamic vinegar samples for OTA contamination and found that only three out of them contained OTA at 102–252 ng/l (Table 6.5), and that the levels in the other samples were lower, at 8–46 ng/l.

## 6.5. OTHER *ASPERGILLUS* MYCOTOXINS

### 6.5.1. CYCLOPIAZONIC ACID (C<sub>20</sub>H<sub>20</sub>N<sub>2</sub>O<sub>3</sub>)

This mycotoxin, which is an indole-derived compound, was first discovered in 1968 as a metabolite of *Penicillium cyclopium* in groundnuts (Holzapfel, 1968). However, some strains of *A. flavus* are also important producers of this mycotoxin (Trucksess et al., 1987). Similarly to aflatoxins, cyclopiazonic acid has also been detected in both the mycelium and the sclerotia of *A. flavus* (Wicklow and Cole, 1982). Other aspergilli, including *A. versicolor*, *A. oryzae*

and *A. tamarii* (but not *A. parasiticus*) have also been recorded as cyclopiazonic acid producers (Dorner, 1983; Dorner et al., 1984).

Cyclopiazonic acid has been added to the category of potentially serious mycotoxins that cause degenerative changes and necrosis in the liver, spleen, pancreas, kidney, salivary glands, myocardium and skeletal muscles (Cole, 1984). It induces sub-acute effects in rats, swine, guinea pigs, dogs and poultry; it affects several organs, and is probably involved together with aflatoxin in the Turkey X Disease in the UK (Scott, 2004). It was originally believed that aflatoxins were responsible for all the toxic effects of *A. flavus*-contaminated groundnuts, but later studies showed that cyclopiazonic acid had an additional severe effect on the muscles and bones of turkeys (Jand et al., 2005). It has been detected in Kodo millet seed in India, where an association with poisoning of humans was suggested (Rao and Husain, 1985).

A study by Gallagher et al. (1978) indicated that *A. flavus* isolated from various sources may produce cyclopiazonic acid more frequently than aflatoxin. Studies by Doster et al. (1996) showed that most of the isolates of *A. flavus* strain L (producers of large sclerotia) which were obtained from fig orchards, and all of those of *A. flavus* strain S (producers of small sclerotia) produced cyclopiazonic acid. None of the strain L isolates produced aflatoxin without also producing cyclopiazonic acid. Several *A. flavus* strains isolated from vine fruits produced cyclopiazonic acid but not aflatoxins (Romero et al., 2005), while all the *A. tamarii* strains isolated from fig orchards were cyclopiazonic acid producers (Doster et al., 1996).

Sobolev et al. (1998) simultaneously determined cyclopiazonic acid, o-methyl sterigmatocystin and versicolorins produced by *A. flavus*, *A. parasiticus* and *A. tamarii*.

### 6.5.2. STERIGMATOCYSTIN (C<sub>18</sub>H<sub>12</sub>O<sub>6</sub>)

This mycotoxin, which is structurally related to aflatoxins, was originally isolated as a metabolite of *A. versicolor*. It was later found to be produced by other species of *Aspergillus*, including *A. flavus* (as a precursor of aflatoxin B<sub>1</sub>), *A. flavipes*, *A. nidulans*, *A. sydowi*, *A. rugulosus* and *A. ustus* (Davis, 1981). It is also produced by species belonging to a number of other fungal genera, including *Chaetomium*, *Bipolaris* and *Emericella* (Davis, 1981). Lisker et al. (1993) studied the mycotoxigenic potential of *A. flavus* strains, and detected sterigmatocystin in 2 percent of the isolates tested.

Sterigmatocystin exhibits weak acute toxicity, mutagenicity, cytotoxicity and carcinogenicity (Terao, 1983). However, its low solubility in water or gastric juices limits its potential to cause human illness (Pitt and Hocking, 1997).

Potential sterigmatocystin-producing aspergilli, such as *A. flavus*, *A. ustus* and *A. versicolor* have been found in wine grapes (Serra et al., 2005), and *A. versicolor* was found to be the source of sterigmatocystin in infected pistachio nuts (Sommer et al., 1976).

### 6.5.3. AFLATREM ( $C_{32}H_{39}NO_4$ )

Aflatrem, which belongs to the tremorgenic toxins, is a natural substance that is known to induce tremors and to elicit acute neurotoxic effects in animals (Valdes et al., 1985). In the mid-1960s it was isolated from mycelial mats and sclerotia produced by *A. flavus*. Similarly to cyclopiazonic acid, it is an indole-derived compound and may be produced by several strains of *A. flavus* grown on various foodstuffs (Luk et al., 1977); it was found in 85 percent of the sclerotia of *A. flavus* strains examined by Wicklow and Cole (1982).

The production of aflatrem by *A. flavus* is often accompanied by aflatoxin production. Purified aflatrem applied in small quantities caused several behavioral changes in animals, and larger doses frequently proved fatal (Gallagher and Wilson, 1978).

### 6.5.4. PENICILLIC ACID ( $C_8H_{10}O_4$ )

This mycotoxin is a small lactone that was originally isolated from *Penicillium puberulum*. It may, however, be produced by several *Penicillium* and *Aspergillus* species. Among the aspergilli capable of synthesizing penicillic acid we find *A. ochraceus*, *A. sulphureus*, *A. melleus* and *A. sclerotiorum*, which are all known to infect figs (Doster et al., 1996; Bayman et al., 2002).

Penicillic acid is active against Gram-negative bacteria (it exhibits antibiotic action against *Echerichia coli*) and Gram-positive bacteria, and also exhibits antiviral, antitumor and cytotoxic effects (Cole and Cox, 1981). The isolation of penicillic acid from a culture filtrate of *A. sclerotiorum* has been recorded, and its high antifungal activity against *Phytophthora* spp. has been demonstrated (Kang and Kim, 2004).

### 6.5.5. CITRININ ( $C_{13}H_{14}O_5$ )

The genus *Aspergillus* includes several citrinin producing species such as *A. candidus*, *A. carneus*, *A. terreus* and *A. flavipes* (Betina, 1989), of which *A. terreus* is a potential pathogen of apples, pears, peaches, grapes, melons and tomatoes (Barkai-Golan, 1980).

Citrinin can be ingested by animals and humans and causes chronic diseases. It is known primarily as a nephrotoxin and several studies have addressed its potential for immunotoxicity (Sharma, 1993). Phytotoxic effects of citrinin have also been reported (Betina, 1989).

### 6.5.6. CO-PRODUCTION OF MYCOTOXINS

The co-production of several secondary metabolites is not rare among the aspergilli. Gallagher et al. (1978) found that out of 54 isolates of *A. flavus*, 28

produced cyclopiazonic acid, 18 produced aflatoxin and 14 produced both. An isolate of *A. flavus*, which simultaneously produced cyclopiazonic acid, aflatrein and aflatoxins, was also described (Richard and Gallagher, 1979). The co-production of two or three toxins increases the toxigenic potential of *A. flavus* and consequently increases the toxic potential of the contaminated commodity.

Co-production of sterigmatocystin with aflatoxin and OTA was recorded in raisins infected by selected strains of *A. flavus* (Youssef et al., 2000), and co-occurrence of OTA and aflatoxin B<sub>1</sub> was reported in dried figs in Turkey by using a single extraction method for OTA (Senyuva et al., 2005).

Bamba and Sumbali (2005) reported that simultaneous production of cyclopiazonic acid and aflatoxin B<sub>1</sub> by *A. flavus* followed infection of sour lime; many of the *A. flavus* isolates were found to produce larger quantities of cyclopiazonic acid (250 to 2501 ng/g) than of aflatoxin (141 to 811.7 ng/g) in the lime tissues. In cases of toxicosis associated with *A. flavus*, it often has been assumed that toxic effects are due to contamination by aflatoxin, and the contribution of cyclopiazonic acid was largely ignored. However, it has recently been suggested that in cases of mycotoxicosis resulting from colonization by *A. flavus*, an effort should be made to quantify both aflatoxin and cyclopiazonic acid, rather than assuming that all the toxic effects are due to aflatoxin alone (Trucksess, 2006).

Co-occurrence of cyclopiazonic acid and tenuazonic acid was reported in Brazilian samples of tomato pulp and tomato puree (da Motta and Soares, 2001), and co-occurrence of citrinin and patulin was recorded in apples with rotten spots (Martins et al., 2002).

## 6.6. FUTURE TRENDS

In light of the high levels of aflatoxins found in susceptible commodities, such as dried figs and pistachios, it is important to continue monitoring their occurrence as a basic step in estimating the level of contamination and assessing preventive methods.

Since OTA is known to have carcinogenic, teratogenic, nephrotoxic and immunosuppressive effects, it raises major concerns for human health. Several studies have emphasized that the ochratoxigenic species *A. carbonarius* is most likely to occur in grapes intended for wine production that are grown in vineyards with climates suitable for fungal development. In order to reduce the risk of occurrence of mycotoxigenic aspergilli in grapes, and the subsequent accumulation of mycotoxins in the wine, only grapes in good condition, which will prevent fungal penetration and development, should be used.

Studies should be carried out to evaluate the effects of improved agricultural and processing techniques on fruit contamination. Emphasis should be placed on both preventive and curative treatments that would lead to the suppression of ochratoxigenic fungal populations in wine-making regions and thus would protect consumers against the risk of exposure to the mycotoxins.

Although a considerable amount of information on OTA in grapes and their products has recently been accumulated, there are some gaps in our knowledge, regarding, for instance, possible year-to-year variations in the level of OTA in these products.

Since accumulation of OTA contamination during the drying of vine fruits may be influenced by the prevailing weather conditions and therefore by the drying rates, safe humidity and temperature regimes during the pre- and postharvest periods are required for developing effective management strategies based on ecological criteria (Magan and Aldred, 2005).

Recent molecular studies indicated that the identification of the biosynthesis genes responsible for mycotoxin production is essential, not only to enable the evolution of mycotoxin biosynthesis in toxigenic aspergilli, but also to support the development of specific gene probes for detection of mycotoxin-producing fungi in agricultural products, and particularly in grapes. Elucidation of the genetic background of the biosynthesis of *Aspergillus* mycotoxin has, therefore, become one of the important future trends in the control of ochratoxigenic pathogens.

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# Penicillium Mycotoxins

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## ABSTRACT

*Penicillium expansum*, the cause of the destructive blue mold rot of pome and stone fruits, is a major concern for human health, because of the production of patulin during pathogenesis. Human exposure is primarily via consumption of infected apple juices and other apple-based products. However, patulin occurrence has been reported in juices of other fruits, such as pears, grapes, cherries, apricots and oranges. The factors affecting *P. expansum* growth and patulin formation include fungal strain, host fruit cultivar, and storage conditions, especially the storage atmosphere. In the present chapter the worldwide occurrence of patulin in fruit products was recorded, with special attention being drawn to patulin contamination in infant apple products, and to comparisons between its levels in: organic fruits versus conventional ones, industrial versus hand-made products, and cloudy versus clear apple juices.

The production of other secondary metabolites of *P. expansum*, such as chaetoglobosins, communesins, roquefortine C and expansolides, have been recorded in a variety of fruits and vegetables. Analysis of apple products clarified that a patulin-negative sample does not always indicate absence of toxic metabolites, and raised the suggestion that chaetoglobosin A might be a better indicator than patulin or might serve as an additional indicator for the presence of *P. expansum* in the fruit.

The ability of a wide range of *Penicillium* species associated with fruits and vegetables, to produce mycotoxins or other secondary metabolites, has been exhibited. *Penicillium* mycotoxins other than patulin, including ochratoxin A, citrinin, penicillic acid, cyclopiazonic acid and penitrem, as well as the co-occurrence of different mycotoxins, have been discussed.

## 7.1. INTRODUCTION

*Penicillium* species are frequently associated with spoilage of foods and feeds, and are therefore of great economic importance. The majority of *Penicillium* species can easily be isolated from the soil, where they take part in the decomposition of organic substances; they are also among the most common airborne fungi (Gregory, 1973), capable of inducing allergic reactions in sensitive patients.

Being common airborne fungi, *Penicillium* spp. can easily be isolated from surfaces of healthy plants, including fruits and vegetables. Several *Penicillium* species are among the most common agents of postharvest diseases and they attack a wide range of fruits and vegetables. *Penicillium expansum*, the major causal agent of the blue mold rot of apples, pears and other deciduous fruits, is an example of the destructive postharvest pathogens that cause a large part of the economic losses that occur during storage and shipment (Barkai-Golan, 2001).

However, in addition to losses they cause in many fruits and vegetables, various *Penicillium* species that are involved in postharvest decay of fruits and vegetables may produce a variety of mycotoxins – secondary metabolites, toxic to animals and humans who consume contaminated foods – during their life cycle in the host fruits (Abramson, 1997; Andersen and Frisvad, 2004; Andersen et al., 2004). These include: patulin, ochratoxin A, citrinin, penicillic acid, cyclopiazonic acid, citreoviridin, penitrem, PR-toxin; and other secondary metabolites, such as chaetoglobosins, communesins, roquefortine C, expansolides, janthitrems, paxillines and others, which may elicit toxic responses in humans and animals. The most common mycotoxin of *P. expansum* is patulin, which is a relatively small molecule that also may be produced by other *Penicillium* species, as well as by various *Aspergillus* species and fungi of other genera.

*Penicillium* is a large genus with 150 recognized species of which 50 or more are of common occurrence (Pitt, 1988). Since the classic *Manual of the Penicillia* by Raper and Thom (1949) the classification of the genus *Penicillium* has undergone re-examination on the basis of several new taxonomic criteria, among which the secondary metabolites produced are important (Frisvad and Filtenborg, 1990; Pitt and Leistner, 1991; Frisvad and Samson, 2004; Paterson et al., 2004).

The present chapter focuses on mycotoxigenic penicillia associated with postharvest diseases, the mycotoxins produced by them in fruits and vegetables, and their derived products.

## 7.2. SIGNIFICANCE OF *PENICILLIUM* SPECIES AS PATHOGENS OF FRUITS AND VEGETABLES

*Penicillium cyclopium* and *P. expansum* are both common pathogens of harvested fruits. Among the host fruits, harvested grapes, followed by harvested tomatoes were found to be most sensitive to *Penicillium* species, whereas pepper and eggplant showed resistance towards most of the penicillia (Barkai-Golan, 1974).

*Penicillium expansum*, known as the causal agent of the blue mold rot in pome and stone fruits, attacks apples, pears, plums, peaches, apricots, cherries, blackcurrants, grapes, melons and strawberries (Snowdon, 1990; Larsen et al., 1998). Fungal penetration into the apple is associated with fruit that has been damaged by preharvest factors, such as insects and storms (Vismer et al.,

1996), or through wounds and bruises inflicted during harvesting and handling (Barkai-Golan, 2001). Penetration can also take place via lenticels under favorable conditions (Baker and Heald, 1934) or at the site of infection by other pathogens, such as species of *Gloeosporium* and *Mucor* (Snowdon, 1990).

Since decay development is favored by high humidity, blue mold rot becomes a problem in fruits stored or shipped in plastic film liners (Hall and Scott, 1989). It is important to know that the fungus can grow at 0°C and that decay can progress, albeit slowly, at this temperature, and can be spread from diseased to sound fruits, by contact between them, during months of cold storage. When the fruits are transferred to warmer shelf-life conditions, rapid development takes place.

Several of the penicillia occurring on wine grapes in Portugal occur as early as the young berry (pea berry) stage, with their number increasing towards harvest (Serra et al., 2005). These include *P. aurantiogriseum*, *P. brevicompactum*, *P. citrinum*, *P. crustosum*, *P. expansum*, *P. funiculosum*, *P. glabrum*, *P. griseofulvum*, *P. purpurogenum*, *P. thomii*, and others.

In a study on fungal population in wine grapes in France, Sage et al. (2002) reported on the occurrence of several different *Penicillium* species, of which *P. brevicompactum* was the most common. In another heavily molded sample, *P. thomii* was the dominant species, and other species, such as *P. chrysogenum*, *P. citrinum*, *P. griseofulvum* and *P. purpurogenum*, were recorded infrequently. The dominance of *P. thomii* in harvested grapes was also reported in two grape-growing areas in Italy (Battilani et al., 2003). In a later study in French vineyards with a variety of climatic conditions the following species were reported by Bejaoui et al. (2006): *P. brevicompactum*, *P. expansum*, *P. spinulosum*, *P. glabrum*, *P. crustosum*, *P. citrinum*, and others. *Penicillium verrucosum*, which has frequently been isolated from cereals, in which it causes ochratoxin contamination in temperate climates, was not isolated from grapes in Italy (Battilani et al., 2003), France (Sage et al., 2002; Bejaoui et al., 2006), or southern Spain (Bellí et al., 2004).

*Penicillium* species are common pathogens of stored grapes. These include *P. aurantiogriseum*, *P. brevicompactum*, *P. chrysogenum*, *P. citrinum*, *P. crustosum*, *P. cyaneo-fulvum*, *P. cyclopium*, *P. decumbens*, *P. frequentans*, *P. glabrum*, *P. puberulum*, *P. stoloniferum*, *P. viridicatum*, among others (Barkai-Golan, 1974; Snowdon, 1990; Benkhemmar et al., 1993).

Several *Penicillium* species, such as *P. brevicompactum*, *P. cyclopium*, *P. puberulum*, *P. expansum*, *P. cyaneo-fulvum*, *P. citrinum*, *P. chrysogenum*, *P. olsonii*, and *P. tularense* may infect stored tomatoes naturally (Barkai-Golan, 1974; Harwig et al., 1979; Andersen and Frisvad, 2004).

The dominant toxigenic penicillia on apples were found to be *P. crustosum* and *P. expansum*, whereas the dominating *Penicillium* species on cherries was *P. expansum* (Andersen and Thrane, 2006). *P. expansum*, the causal agent of the blue mold rot of apples and pears, also infects stone fruits such as nectarines, peaches, apricots, cherries, and plums, as well as melons and strawberries (Snowdon, 1990). Other *Penicillium* species, such as *P. cyclopium*, *P. viridicatum*, *P. verrucosum* and *P. funiculosum* may occasionally cause the blue mold

rot on apples, pears, pineapples and melons (Snowdon, 1990; Barkai-Golan, 2001).

Investigating fungal contamination of stored and freshly harvested vegetables, Lugauskas et al. (2005) found that *P. expansum*, *P. corymbiferum*, *P. granulatum*, *P. nalgiovense* and *P. verrucosum* were common on carrots, *P. expansum* was common on onions, and *P. expansum* and *P. funiculosum* were commonly found on cabbages. Several *Penicillium* species, such as *P. corimbiferum*, *P. cyclopium*, *P. expansum*, *P. purpurescence*, and *P. viridicatum* may be responsible for rots on onions and garlics (Snowdon, 1992; Lugauskas et al., 2005; Overy et al., 2005). Lugauskas et al. (2005) quantitatively confirmed the ability of *P. expansum* to grow and produce mycotoxin on potatoes.

### 7.3. MYCOTOXINS AND OTHER TOXIC METABOLITES OF *PENICILLIUM* SPECIES

Abramson (1997) divided *Penicillium* mycotoxins into four classes, according to their significance in term of agro-economics and human health: those implicated in human diseases (Class A); those implicated in animal diseases (Class B); those found in foods and feeds (Class C); and those exhibiting oral toxicity and that could be produced in fungal cultures (Class D). However, these classes overlap, and a single mycotoxin, such as ochratoxin A, could be implicated in livestock toxicosis and therefore would belong to Class B, but it might also be found in foods and feeds (Class C) and could be produced in fungal cultures (Class D) (Abramson, 1997).

The major *Penicillium* mycotoxin associated with fruit and vegetable decay, and the most studied one, is patulin. Other such mycotoxins include ochratoxin A, citrinin, penicillic acid, cyclopiazonic acid and penitrem. Other secondary metabolites of *Penicillium* species also fall within the category of *Penicillium* toxic compounds. They include chaetoglobosins, communesins, roquefortine C and expansolides, all produced by *P. expansum*; roquefortine C and secalonic acid produced by *P. chrysogenum*; janthitrems, paspalinine, paxillines and *O*-acetopaxilline, produced by *P. tularensis*; and verrucolone, produced by *P. olsonii* (Andersen and Frisvad, 2004; Andersen et al., 2004).

Mycotoxigenic penicillia associated with fruit and vegetable decay, along with mycotoxins and other secondary metabolites produced by them, are given in Table 7.1.

Several *Penicillium* species listed in Table 7.1 are generally capable of producing a number of potent mycotoxins. These include *P. expansum* which, in addition to patulin, may produce citrinin, penicillic acid, cyclopiazonic acid, and other secondary metabolites such as chaetoglobosins, communesin B, roquefortine C and expansolides. *Penicillium viridicatum* may similarly produce several mycotoxins (Table 7.1). The mycotoxin profile of *P. chrysogenum* includes, in addition to patulin, ochratoxin A, penicillic acid, cyclopiazonic acid and roquefortine C, *Penicillium olsonii* produces

TABLE 7.1 *Penicillium* species associated with fruits and vegetables and the mycotoxins or other secondary metabolites they are capable of producing.

<i>Penicillium</i> species	Mycotoxins and other secondary metabolites	References
<i>P. aurantiogriseum</i>	Chaetoglobosin A Penicillic acid Verrucosidin	Veselý et al., 1995 Frisvad et al., 2004 Frisvad et al., 2004
<i>P. brevicompactum</i>	Patulin Mycophenolic acid	Paterson et al., 2004 Cole and Cox, 1981
<i>P. chrysogenum</i>	Penicillic acid Ochratoxin A Patulin Cyclopiazonic acid Secalonic acid Roquefortine C PR-toxin	Leistner and Pitt, 1977 Turner and Aldridge, 1983 Moss, 1987 Cole, 1984 Andersen and Frisvad, 2004 Andersen and Frisvad, 2004 Frisvad et al., 2004
<i>P. citrinum</i>	Citrinin Citreovertin	Cole and Cox, 1981 Leistner and Eckardt, 1979
<i>P. citreoviride</i>	Citreovertin	Cole and Cox, 1981
<i>P. commune</i>	Ochratoxin A Cyclopiazonic acid	Turner and Aldridge, 1983 Frisvad et al., 2004
<i>P. corymbiferum</i>	Patulin Roquefortine	Lugauskas et al., 2005 Lugauskas et al., 2005
<i>P. crustosum</i>	Penitrem A Cyclopiazonic acid Roquefortine C	de Jesus et al., 1983 Cole, 1984 Andersen and Thrane, 2006
<i>P. cyaneo-fulvum</i>	Patulin	Turner and Aldridge, 1983
<i>P. cyclopium</i>	Penicillic acid Cyclopiazonic acid Patulin Ochratoxin A Viomellein Xanthomegnin Vioxanthin Roquefortine Rugulosin	Leistner and Pitt, 1977 Cole, 1984 Leistner and Pitt, 1977 Turner and Aldridge, 1983 Stack and Mislivec, 1978 Stack and Mislivec, 1978 Frisvad et al., 2004 Turner and Aldridge, 1983 Turner and Aldridge, 1983
<i>P. discolor</i>	Chaetoglobosins	Frisvad et al., 1997
<i>P. expansum</i>	Patulin Citrinin Cyclopiazonic acid Penitrem A Chaetoglobosin A, C Communesin B Roquefortine C Expansolides A, B	Harwig et al., 1979 Harwig et al., 1973 Bridge et al., 1989 Bridge et al., 1989 Larsen et al., 1998 Andersen et al., 2004 Andersen et al., 2004 Andersen et al., 2004
<i>P. granulatum</i>	Patulin	Lugauskas et al., 2005
<i>P. griseofulvum</i> <sup>a</sup>	Patulin Griseofulvin Cyclopiazonic acid	Frisvad et al., 2004 Cole and Cox, 1981 Cole, 1984
<i>P. nalgiovense</i>	Cyclopiazonic acid	Lugauskas et al., 2005
<i>P. nordicum</i> <sup>b</sup>	Ochratoxin A Viridic acid	Larsen et al., 2001 Frisvad et al., 2004

(Continued)



TABLE 7.1—(Cont'd)

<i>Penicillium</i> species	Mycotoxins and other secondary metabolites	References
<i>P. olsonii</i>	Verrucolone	Andersen and Frisvad, 2004
<i>P. patulum</i> <sup>c</sup>	Patulin	Turner and Aldridge, 1983
	Griseofulvin	Cole and Cox, 1981
<i>P. puberulum</i>	Cyclopiazonic acid	Cole, 1984
<i>P. purpurrescens</i>	Ochratoxin A	Turner and Aldridge, 1983
	Citrinin	Leistner and Pitt, 1977
<i>P. purpurogenum</i>	Rubratoxins	Davis and Richmond, 1984
	Ochratoxin A	Sage et al., 2002
<i>P. roqueforti</i>	Penicillic acid	Leistner and Eckardt, 1979
<i>P. rubrum</i>	Rubratoxins	Davis and Richmond, 1984
	Xanthoviridicatins	Stack et al., 1979
<i>P. rugulosum</i>	Rugulosin	Lugauskas et al., 2005
	Ochratoxin A	Sage et al., 2002
<i>P. thomii</i>	Penicillic acid	Karow et al., 1944
<i>P. tularensense</i>	Paspalinines	Andersen and Frisvad, 2004
	Paxillines	Andersen and Frisvad, 2004
	3-O-acetoxypaxilline	Andersen and Frisvad, 2004
	Janthitrems	Andersen and Frisvad, 2004
<i>P. verrucosum</i> <sup>b</sup>	Ochratoxin A	Abramson, 1997
	Citrinin	Larsen et al., 2001
	Verrucolone	Larsen et al., 2001
	Verrucines	Larsen et al., 2001
<i>P. verrucosum</i>	Penicillic acid	Samson et al., 1976
Var. <i>cyclopium</i>	Ochratoxin A	Samson et al., 1976
<i>P. viridicatum</i> <sup>d</sup>	Penicillic acid	Abramson, 1997
	Ochratoxin A	van Walbeek et al., 1969
	Cyclopiazonic acid	Cole, 1984
	Citrinin	Krogh et al., 1970
	Rubrosulphin	Stack et al., 1977
	Viomellein	Stack et al., 1977
	viioxanthin	Frisvad et al., 2004
	Viopurpurin	Stack et al., 1977
	Viridic acid	Frisvad et al., 2004
	Viridicatin	Cole and Cox, 1981
	Xanthomegnin	Stack et al., 1977
	Xanthoviridicatins	Stack et al., 1979
	Viridicatum-toxin	Kabuto et al., 1976

<sup>a</sup> Formerly *P. urticae*.  
<sup>b</sup> The species *P. verrucosum* and *P. nordicum* were considered to belong to the series *Verrucosa* subgenus *Penicillium*, although these two ochratoxin-producing fungi are biosynthetically and ecologically very different (Larsen et al., 2001). Ochratoxin-producing penicillia are gathered within *P. verrucosum* and its chemotypes (Abramson, 1997; Pitt, 1987).  
<sup>c</sup> *P. patulum* is currently regarded as *P. griseofulvum*.  
<sup>d</sup> The taxonomic status of ochratoxin producing-penicillia has changed often during the past century and for years they were classified as *P. viridicatum*. Later studies by Pitt (1987) and Frisvad and Filtenborg (1990) considered chemotypes within *P. viridicatum* or *P. verrucosum* and agreed that *P. verrucosum* is the only ochratoxin-producing species of the genus *Penicillium*. However, both scientists retained two chemotypes within the species, of which chemotype II is the only one capable of producing citrinin (Larsen et al., 2001).

verrucolone, and *P. tularensis* produces paspalinines, paxillines and janthitrems (Andersen and Frisvad, 2004).

However, *P. digitatum* and *P. italicum*, which are major postharvest pathogens of harvested citrus fruits, have not been included among the mycotoxigenic penicillia, although several metabolites, such as isomers of methoxyphenol, phenyl methoxypropylphenol, octadecanoic acid and others, have been identified in toxic extracts from *P. digitatum* and *P. italicum* isolates (Tantaoui-Elaraki et al., 1994).

## 7.4. PATULIN

### 7.4.1. FUNGI ASSOCIATED WITH PATULIN PRODUCTION

Patulin ( $C_7H_6O_4$ ) is a relatively uncomplex lactone, derived mainly from species of *Penicillium*, *Aspergillus*, *Paecilomyces* and *Byssosclamyces* (Cole and Cox, 1981). It also can, however, be produced by species of *Stemphylium*, *Alternaria*, *Fusarium*, *Trichoderma*, *Trichothecium*, *Mucor* and *Phialophora* (Steinman et al., 1989; Laidou et al., 2001), which are all capable of inducing postharvest diseases in fruits and vegetables (Snowdon, 1990; Barkai-Golan, 2001). The major producer of patulin in nature is *P. expansum*, commonly known as the blue mold pathogen of apples and other deciduous fruits. Following cloning and molecular characterization of *P. expansum* genes under patulin-permissive conditions, White et al. (2006) showed that the regulation of patulin biosynthesis in *P. expansum* is mediated at the level of gene transcription.

Other *Penicillium* species also have been reported as patulin producers; among them we find several fruit pathogens, such as *P. cyclopium*, *P. chrysogenum* and *P. cyaneo-fulvum* (Barkai-Golan, 1974). Some *P. griseofulvum* strains have been found able to produce patulin at levels higher than those produced by *P. expansum* strains, on suitable culture media (Dombrink-Kurtzman and Blackburn, 2005).

The wide distribution of mycotoxigenic penicillia among fungi pathogenic to fruits and vegetables raises the question of whether mycotoxins fill a role in the pathogenicity of harvested crops, in addition to their role in animal diseases (Desjardins and Hohn, 1997). Patulin is a cyclic tetraketide known to have phytotoxic activity, and the gene encoding 6-methylsalicylic acid synthase, a polyketide synthase involved in patulin biosynthesis, has been isolated from the patulin-producing species, *P. patulum* and *P. urticae*, both currently designated as *P. griseofulvum* (Beck et al., 1990; Wang et al., 1991).

### 7.4.2. TOXIC EFFECTS OF PATULIN

Patulin is highly toxic to plant and animal cells, and can react with terminal sulfhydryl groups of proteins and polypeptides present in food (Wouters and

Speijers, 1996). It was found to inhibit protein and RNA synthesis, and was shown to produce persistent breaks in single and double strands of DNA in *Escherichia coli* (Lee and Roschenthaler, 1986). A later study revealed its ability to cause oxidative damage to the DNA in human cells (BingHui et al., 2003). It may also inhibit the activity of numerous enzymes, mainly as a consequence of its strong affinity to sulfhydryl groups (Wouters and Speijers, 1996) and has an inhibitory effect on several biochemical parameters, such as AT-Pase, alkaline phosphatase, aldolase and hexokinase activity (Speijers, 2004).

Patulin had mutagenic, genotoxic, immunotoxic and neurotoxic effects on rodents (Ritieni, 2003) and teratogenic effects on chickens (Ciegler et al., 1977). A study on the genotoxic effects of patulin showed that they could differ qualitatively and quantitatively in different mammalian cells (Lehmann et al., 2003). The mutagenicity of patulin in cultured Chinese hamster cells was especially pronounced in cells with low glutathione concentration (Schumacher et al., 2005).

Exposure of humans to patulin via consumption of infected products may result in severe toxicosis, including mutagenic, teratogenic, hepatotoxic, nephrotoxic, neurotoxic and genotoxic effects; its acute effects include nausea, vomiting and other gastrointestinal traumas that accompany kidney damage (Ciegler et al., 1977; Brackett and Marth, 1979; Hopkins, 1993). At high doses patulin was found to exhibit immunosuppressive properties (Sharma, 1993).

Although possible carcinogenic properties have been attributed to patulin by Dickens and Jones (1961), this interpretation was found to be based on an inadequate study; recent safety assessments have concluded that patulin is not carcinogenic, and a tolerable daily intake has been designated (Speijers, 2004). On the other hand, the recently reported ability of patulin to cause gene mutations in mammalian cells might have a bearing on its carcinogenicity (Schumacher et al., 2005).

Investigating the fate of patulin in human blood that followed the consumption of a commercial patulin-containing apple juice, Rychlik (2005) found that the mycotoxin was rapidly degraded before it reached tissues other than the gastrointestinal tract. The degradation was attributed to a reaction with glutathione, in light of the detection of several glutathione-patulin adducts in the blood.

### 7.4.3. PATULIN PRODUCTION IN FRUITS

#### 7.4.3.1. Apples, pears and other fruits

Patulin is a common food contaminant that has been detected in a wide range of fruits and their products. Although it can be produced by a variety of *Penicillium* species its major sources in the market are apples and, to a lesser extent, pears infected with *P. expansum*. After harvest, apples and pears are usually stored under refrigeration, which extends their shelf lives to several

weeks or months, depending on the cultivar. However, *P. expansum* continues to grow and produce patulin in many apple and pear cultivars during cold storage (Barkai-Golan, 2001).

Patulin frequently has been recorded in other harvested fruits. Its production in grapes has been associated with moldy berries (Abrunhosa et al., 2001) and with fruits infected during prolonged cold storage (Scott et al., 1977); it has been detected in peaches, apricots, cherries, blackcurrants and olives (Lovett et al., 1974; Larsen et al., 1998; de Sylos and Rodriguez-Amaya, 1999; Arici, 2000), and an outbreak of diarrhea in children in Sweden was attributed to the consumption of blueberries with a high patulin content (Berg et al., 1995). In addition to patulin in a variety of fresh fruit this mycotoxin has also been recorded recently in dried figs (Karaca and Nas, 2006).

#### 7.4.3.2. Factors affecting patulin production in the fruit

Several studies revealed that fruit cultivars may differ in their susceptibility to *P. expansum* rotting and to patulin contamination (Spotts and Mielke, 1999). Martins et al. (2002) studied apples belonging to seven cultivars in Portugal that exhibited small rotten areas. They found patulin concentrations up to 80.5 mg/kg in cv. Richard, in which it was frequently recorded together with lower concentrations of citrinin. These results showed the possible risk of human exposure to patulin through the consumption of apple juices or jams manufactured from apples with small rotten spots. High levels of patulin (110 mg/kg) were recorded by Ostry et al. (2004) in cv. Gloster, which is processed for baby food in the Czech Republic.

The specific *P. expansum* strain may be an important factor in its pathogenicity and in its ability to synthesize patulin in the fruit. Studying patulin contamination in apples, Sommer et al. (1974) found that different *P. expansum* strains produced differing levels, ranging from 2 to 100 µg/g, and that the levels were not related to the pathogenic vigor of the *P. expansum* strains. This led to the suggestion that patulin probably does not play a role in *P. expansum* pathogenicity. Beretta et al. (2000) similarly found that the patulin content in apples was not always correlated with the extent of the rotten areas, since very high levels have sometimes been detected in fruits with small affected areas.

McCallum et al. (2002) found extensive differences among *P. expansum* isolates, in terms of patulin production and fungal growth in apple ciders. Genetic variations may have been the ultimate cause of these differences, but environmental factors such as temperature and pH, as well as the storage duration, could contribute greatly to fungal growth and patulin production. The minimum temperature range for patulin production by *P. expansum* in apples is 1–4°C, depending on the apple variety (Northolt et al., 1978). Thus, patulin levels in Macintosh apples stored at 4°C were substantially lower than in those stored at 15 or 24°C.

Studying patulin production by different strains of *P. expansum* in apples and pears, Paster et al. (1995) demonstrated the triple effect of pathogen, host and storage temperature. All the *P. expansum* strains examined produced

patulin in Spadona pears at 3, 6 and 17°C, but only two of them formed the toxin at the extreme temperatures tested: 0 and 25°C.

*Penicillium expansum* isolates originating from locations across Ontario differed in the levels of patulin produced, which ranged from 0 to >6 µg/g, as measured in dry mycelia (McCallum et al., 2002). The final dry mycelial growth was negatively correlated with decreases in the pH value, and the latter directly affected patulin production, although a higher mycelial growth rate did not necessarily lead to a higher level of patulin production, on a dry-weight basis (McCallum et al., 2002). It was suggested that the fungus itself might acidify the culture medium by producing secondary metabolites and organic acids, and thus lower the pH to the point at which patulin is most stable (pH < 3.5) (Tsao and Zhou, 2000).

Several intrinsic characteristics of the apple were found to affect *P. expansum* growth and patulin production (Baert et al., 2004). Fungal growth on apple purée agar resulted in decreased levels of sucrose, glucose and fructose, and the production of lactic acid by the fungus reduced the pH of the medium. In its first phase of growth, *P. expansum* exhibited an exponential increase in the production of patulin, and the rate of increase was enhanced as the pH rose. No correlation between the sugar content of the apples and patulin production by the fungus was recorded. Beer and Amand (1974) mentioned another factor – the fruit firmness – that affected patulin contamination, and showed that the amount of patulin produced in *P. expansum*-inoculated apples was inversely proportional to the firmness of the cultivar.

The procedure used for preservation of *P. expansum* may affect the activities of the strain, including mycotoxin production (Santos et al., 2002). Santos et al. (2002) studied the effects of four culture preservation methods – maintenance at 4°C, preservation under mineral oil, silica gel storage, and centrifugal freeze-drying – on mycotoxin production by *P. expansum* and showed that whereas citrinin could be produced by cultures subjected to any of the above preservation techniques, patulin profiles seemed to be strain-specific and dependent on the preservation technique. Higher consistency for patulin detection was found in cultures preserved under silica-gel storage and following freeze drying. Furthermore, patulin could be detected in cultures that had undergone preservation, even if none had been produced originally (Santos et al., 2002).

#### 7.4.3.3. Modified or controlled atmosphere storage of apples and pears

Another environmental factor that affects mycotoxin production is the storage atmosphere. Elevating the CO<sub>2</sub> level and reducing the O<sub>2</sub> tension to produce a modified atmosphere (MA) or a controlled atmosphere (CA) are known means of prolonging the postharvest life of apples and pears. Such atmospheres retard the senescence processes of the fruit and suppress postharvest disease development, either directly by suppressing pathogen growth, or indirectly by maintaining the infection resistance of the host by enhancing its physiological condition (Barkai-Golan, 1990). Storage of ‘Golden Delicious’ apples under a CA tolerated by most fruits (2–8 percent CO<sub>2</sub> and 2–3 percent O<sub>2</sub>) was found to

suppress both *P. expansum* growth and patulin accumulation in the fruit (Sommer et al., 1974), and juice made from apples subjected to CA storage (3 percent O<sub>2</sub>, 1–3 percent CO<sub>2</sub> and >90 percent relative humidity) contained much less patulin (500 µg/l) than that made from air-stored apples (2000–3000 µg/l) (Lovett et al., 1975).

Elevated CO<sub>2</sub> levels inhibited both the growth of *P. patulum* and its ability to produce patulin (Paster and Lisker, 1985). Patulin production was inhibited when the CO<sub>2</sub> was elevated to above 10 percent while the O<sub>2</sub> level was maintained at 20 percent. Storing apples under a CA containing 3 percent CO<sub>2</sub> and 2 percent O<sub>2</sub> – a composition frequently used to extend the postharvest life of apples – Paster et al. (1995) found an approximately 30 percent reduction in the weight of infected tissues compared with that in air-stored fruits. Under these conditions patulin production by three strains of *P. expansum* was totally inhibited.

Although high CO<sub>2</sub> (>3 percent) atmospheres acted as a fungistatic treatment for apples stored at 0°C, excessively high levels of CO<sub>2</sub> (>8 percent) negatively affected the quality of some apple cultivars (Sitton and Patterson, 1992).

The advantage of storing apples under a very-low-O<sub>2</sub> atmosphere (0.75 percent O<sub>2</sub>) compared with storage at higher O<sub>2</sub> levels (1.0–1.25 percent O<sub>2</sub>) was shown by Johnson et al. (1993), who found that apples subjected to storage under a 0.75 percent-O<sub>2</sub> atmosphere from which ethylene was removed were firmer and showed reduced incidence of *Penicillium* rot. Such a result could have been achieved by the reduction both of the primary infection and of the secondary infection caused by contact between infected and sound fruits (Barkai-Golan, 2001).

The effects of a CO<sub>2</sub>-enriched atmosphere on pathogen development and patulin production in polyethylene (PE)-packaged apples were studied by Moodley et al. (2002) in ‘Granny Smith’ apples that had been inoculated with *P. expansum* and held at 25°C. The PE film, which allowed CO<sub>2</sub> to accumulate and O<sub>2</sub> to be depleted within the packaging system, was very effective in suppressing fungal growth. Patulin production was almost completely inhibited by gas combinations including 58 percent CO<sub>2</sub> with 42 percent N<sub>2</sub>, 48 percent CO<sub>2</sub> with 52 percent N<sub>2</sub> and 88 percent CO<sub>2</sub> with 12 percent N<sub>2</sub>. In contrast, packaging apples in polypropylene (PP) did not inhibit fungal growth in any of the atmospheres tested. The structural differences between PP and PE films, in the degrees of polymeric branching and the pore sizes, allowed much lower water vapor transmission rates through PP packaging than through PE, so that at the end of the incubation period more moisture accumulated in the former, resulting in enhanced fungal growth (Moodley et al., 2002).

#### 7.4.4. ABILITY OF PATULIN TO PENETRATE HEALTHY FRUIT TISSUE

Patulin contamination is primarily confined to the decayed part of the fruit and can usually be removed before processing, but some apple cultivars, such as ‘Bramley’ have an open core where infection may occur without being

apparent on the fruit surface. In such cases damage to the fruit is not easily observed and infected tissue might not be removed before processing (Moss, 2002).

Several studies indicated that patulin is capable of penetrating up to several millimeters into the surrounding healthy tissue (Taniwaki et al., 1992; Beretta et al., 2000; Laidou et al., 2001; Ostry et al., 2004). The ability of patulin to spread into non-infected, apparently healthy areas of *P. expansum*-infected fruit has been demonstrated by Martins et al. (2002) in several apple cultivars. A study with inoculated pears similarly showed that the removal of the rot along with nearly 10 mm of the surrounding fruit tissues could not protect consumers from this mycotoxin (Laidou et al., 2001). In other words, the absence of rots in the flesh of such pears does not necessarily mean absence of patulin or of the toxigenic fungus.

Interest in the presence of patulin in fruit juices and other fruit-based products has markedly increased during recent years, and reports of its occurrence cover countries in Europe and North and South America, as well as South Africa, Japan, and others.

## 7.4.5. PATULIN IN FRUIT PRODUCTS

### 7.4.5.1. Patulin in apple-based products

The apple cultivar, the quality of the fruit used for processing, the presence of factors that reduce patulin stability or enhance its degradation (Drusch et al., 2007), along with the analytical method used for toxin detection and quantification, may markedly affect the levels of patulin recorded in the analyzed juice samples.

In France, 100 percent incidence of patulin was found in apple juices and apple juice concentrates by Bohuon and Drilleau (1980) and by Jelinek et al. (1989), at levels up to 610 µg/kg. In studying Italian fruit juices Vallettrisco et al. (1983) found patulin (at levels up to 60 ng/ml) in each of the five samples of apple juice examined. In apple juices from Turkey Karadenizim and Eksi (1997) recorded 100 percent incidence of patulin, at levels up to 75 µg/l, and Gökmen and Acar (1998) recorded levels up to 376 µg/l. However, in their study on apple juice concentrates, Gökmen and Acar (2000) emphasized the year-to-year variations in patulin concentrations. High levels (up to 732.8 µg/l) of patulin, with 60 percent of samples contaminated, were reported in apple juice from Turkey by Yurdun et al. (2001), and in apple juice and apple juice concentrate from Iran (up to 285.3 and 148.8 µg/l, respectively) with 33 and 56 percent, respectively, of samples contaminated (Cheraghali et al., 2005).

Patulin levels of 10–170 µg/l were recorded in Spanish apple juice (Prieta et al., 1994) and levels below the recommended limits were recorded in apple juice from Sweden (Thuvander et al., 2001), from Cuba (Fernandez-Trejejo et al., 2001) and from the Basque Country (Armentia et al., 2000). Low levels



and low incidence of patulin were recorded in apple juices surveyed in Brazil by de Sylos and Rodriguez-Amaya (1999) and by Prado et al. (2000), and no patulin was recorded in 14 apple juice samples from Brazil surveyed by Iha and Sabino (2006). Levels up to 10 µg/l were reported in Japan for local juice (Watanabe and Shimizu, 2005).

*a. Infant apple products.* Several studies focused on patulin contamination in fruit products for infants or children. Studies by Brown and Shephard (1999) in South Africa showed that 23 percent of the apple juices and 29 percent of the infant apple products contained patulin at 10–45 and 5–20 ppb, respectively. All the samples of baby foods analyzed in Italy by Beretta et al. (2000) had patulin content below the safe limit (75 µg/kg), with maximum levels of 6.39 and 5.97 µg/kg for conventional and ecological (only a few, selected pesticides) products, respectively. In another study on patulin content in baby food from Italy, two out of 10 apple-based samples were found to be contaminated with patulin at 17.7 and 13.1 µg/l (Ritieni, 2003).

*b. Organic versus conventional apple products.* With the growing public interest in organically produced fruits and vegetables, aroused by the possible freedom from chemical contaminants associated with this mode of production, several studies have been focused on patulin contamination in organic compared with conventional products. Pussemier et al. (2006) emphasized, however, that organic agriculture does not keep within the limits of a production system in which the use of certain chemical inputs, such as fertilizers, and plant protection or veterinary products, is forbidden.

In Italy, Beretta et al. (2000) recorded significantly higher levels of patulin in apple juice from organic than in that from conventional products, with maximum values of 28.4 and 3.03 µg/kg, respectively, and in a later study Piemontese et al. (2005) also found higher incidence of patulin-positive samples and higher levels of patulin contamination in organic than in conventional products. The estimated daily intakes of patulin, per kilogram of body weight, were 0.38 and 1.57 ng from conventional and organic products, respectively, and those for children were higher, reaching 3.41 ng from conventional products and 14.17 ng from the equivalent organic products, but these levels were largely below the provisional maximum tolerable daily intake of 400 ng/kg (Piemontese et al., 2005). In light of these findings, stricter control, prior to their processing, of fruits produced under organic conditions has been suggested.

Boonzaaijer et al. (2005) analyzed the patulin contents of 63 commercial apple products, including both conventional and organic items, available in the Dutch market. With the exception of one sample (organic apple juice), the mycotoxin concentration was lower than the detection limit (25 µg/l or 25 µg/kg) in all the samples. No significant difference was found between the conventional and the organic apple product samples.

Baert et al. (2005) also found no significant difference between conventional and organic apple juice when they evaluated the exposure of young children to patulin through the consumption of different types of apple juice



in Belgium. However, two of the organic apple juice samples contained patulin above the legal limit of 50 µg/l.

*c. Industrial versus hand-made apple products.* In Belgium Tangni et al. (2003) found no statistically significant differences in the mean patulin contamination between hand-made apple juice, with a mean of 14.6 µg/l, and industrial apple juice, with a mean of 7 µg/l. No contaminated samples exceeded the safe patulin level of 50 µg/l. No statistical difference was found between the mean patulin contaminations in clear (7.8 µg/l) and cloudy (10.7 µg/l) apple juices, whether they were industrial or hand-made (Tangni et al., 2003).

*d. Cloudy versus clear apple juices.* Studies by the Ministry of Agriculture, Fisheries and Food (MAFF) in the UK in 1992 reported on one clear juice sample with a patulin content of 118 µg/kg and four cloudy apple juice samples with 59–434 µg/kg. A reduced level of patulin was recorded in cloudy apple juice following clarification, and the solid residue became enriched in patulin (Bissessur et al., 2001). A decrease in patulin recovery was observed when cloudy apple juice was spiked with this mycotoxin during validation of an HPLC-UV method for patulin analysis (Baert et al., 2007). The decrease in patulin recovery was hypothesized to be related to interactions between patulin and the solid components of the cloudy juice. This hypothesis was based on the findings that the solid components of the cloudy apple juice were richer in proteins than the liquid phase (Baert et al., 2007) and emphasized that patulin is capable of binding to proteins (Fliege and Metzler, 2000).

The finding that up to 20 percent of the patulin was bound and could not be detected by the HPLC-UV analysis indicates that the toxin level of the cloudy juice could be underestimated (Baert et al., 2007).

#### 7.4.5.2. Patulin in products of other fruits

Although most studies on patulin occurrence in fruit juices addressed apple juices, the presence of this mycotoxin has also been demonstrated in other fruit juices. In grape juices in Germany patulin levels up to 230 and 5.2 µg/l were recorded by Altmayer et al. (1982) and Rychlik and Schieberle (1999), respectively. In pear, apricot and peach juices from Italy concentrations of 25, 12 and 3 µg/l, respectively, were recorded by Vallettrisco et al. (1983), whereas concentrations of only 0.1–0.2 µg/l were found in sour cherry, blackcurrant and orange juices from Germany (Rychlik and Schieberle, 1999). No patulin was recorded by Mortimer et al. (1985) in grape juice from the UK, or in pear and quince juices from Germany by Lehman and Wald (1990).

Analyses by de Sylos and Rodriguez-Amaya (1999) of fruit juices obtained from apple, grape, pineapple, papaya, guava, banana and mango in Brazil indicated that only one out of 30 samples of the apple juice contained patulin (at 17 µg/l). It was not detected at any of the sound peach samples but was

found in 14 out of 150 samples of spoiled peaches. The results suggested that the fruit used for processing was of good quality. However, since most of the fruit juices had been sulfited, as permitted by Brazilian regulation, the low levels of patulin may also have been due to the sulfur dioxide, which is known to decrease patulin concentration.

Studying the production of mycotoxins by *P. expansum* in blackcurrant and cherry juice, Larsen et al. (1998) found that the fungus was capable of synthesizing extracellular patulin at both 10 and 25°C, and that when unlimited oxygen was available, another secondary metabolite – chaetoglobosin – could be detected extracellularly, along with patulin. Patulin and chaetoglobosins A and C, communesins and expansolides could be detected intracellularly in fungal mycelia under suitable conditions.

Patulin was also found in apple vinegar from Italy at levels below 5 µg/kg (Piemontese et al., 2005). The finding of patulin in apple vinegar suggested that patulin degradation during fermentation might not be complete, so that residual levels could be found in the products.

The occurrences of patulin in fruit juices and other fruit products in various countries are summarized in Table 7.2.

TABLE 7.2 Natural occurrence of patulin in fruit juices and other fruit products.

Commodity	Location	Positive/total patulin- containing fruit	Patulin maximal level µg/l or µg/kg	References
Apple juice	Finland	10/51	72	Lindorth and Niskanen, 1978
Apple juice concentrate	France	27/27	610	Bohuon and Drilleau, 1980
Grape must	Germany	30/64	230	Altmayer et al., 1982
Grape juices and wines	Germany	21/55	230	Altmayer et al., 1982
Jam	Italy	10/20	75	Valletrisco et al., 1983
Apple juice	Italy	5/5	60	Valletrisco et al., 1983
Pear juice	Italy	5/6	25	Valletrisco et al., 1983
Apricot juice	Italy	1/3	12	Valletrisco et al., 1983
Peach juice	Italy	2/6	3	Valletrisco et al., 1983
Apple juice	UK	10/25	56	Mortimer et al., 1985
Grape juice	UK	0/13	–	Mortimer et al., 1985
Apple juice and products	Germany	73/91	402	Ehlers, 1986
Apple juice concentrate	France	27/27	610	Jelinek et al., 1989
Apple cider	France	13/27	300	Jelinek et al., 1989
Apple juice	Germany	0/10	–	Lehmann and Wald, 1990
Quince juice	Germany	0/8	–	Lehmann and Wald, 1990

(Continued)

TABLE 7.2—(Cont'd)

Commodity	Location	Positive/total patulin- containing fruit	Patulin maximal level µg/l or µg/kg	References
Pear juice	Germany	0/2	—	Lehmann and Wald, 1990
Apple and mixed fruit juice	New South Wales	101/241	1130	Burda, 1992
Apple juice, clear	UK	1/17	118	MAFF, 1993
Apple juice, cloudy	UK	4/15	434	MAFF, 1993
Apple juice	Spain	43/100	170	Prieta et al., 1994
Infant apple food	Spain	0/12	—	Prieta et al., 1994
Apple juice	Turkey	44/44	75	Karadenzim and Eksi, 1997
Apple juice	Turkey	215/215	376	Gökmen and Acar, 1998
Apple juice	Germany	10/10	26	Rychlik and Schieberle, 1999
Grape juice	Germany	2/2	5.2	Rychlik and Schieberle, 1999
Sour cherry juice	Germany	1/1	0.2	Rychlik and Schieberle, 1999
Blackcurrant juice	Germany	1/1	0.1	Rychlik and Schieberle, 1999
Orange juice	Germany	1/1	0.1	Rychlik and Schieberle, 1999
Apple juice	Germany	9/9	19.8	Steiner et al., 1999
Pear juice	Germany	0/2	—	Steiner et al., 1999
Apple juice	Brazil	1/30	17	de Sylos and Rodriguez-Amaya, 1999
Apple juice (conventional)	Italy	21/21	3.03	Beretta et al., 2000
Apple juice (organic)	Italy	21/21	28.24	Beretta et al., 2000
Apple baby food (conventional)	Italy	23/23	6.39	Beretta et al., 2000
Apple baby food (ecological)	Italy	23/23	5.97	Beretta et al., 2000
Apple juice with pulp	Italy	10/12	1150	Beretta et al., 2000
Apple juice	Taiwan	59/71	39.9	Lai et al., 2000
Carbonated apple juice	S. Africa	2/4	45	Leggott and Shephard, 2001
Infant apple juice	S. Africa	2/3	20	Leggott and Shephard, 2001
Infant mixed juice	S. Africa	4/7	20	Leggott and Shephard, 2001
Apple products	S. Africa	2/6	10	Leggott and Shephard, 2001
Infant apple purée	S. Africa	0/1	—	Leggott and Shephard, 2001
Apple, pineapple, & passion juices	France	8/44	60	Ake et al., 2001

TABLE 7.2—(Cont'd)

Commodity	Location	Positive/total patulin- containing fruit	Patulin maximal level µg/l or µg/kg	References
Apple juice	Turkey	27/45	732.8	Yurdun et al., 2001
Apple juices & other products	Italy	11/40	74.2	Ritieni, 2003
Apple baby food (organic)	Italy	2/10	17.7	Ritieni, 2003
Apple juice <sup>a</sup>	Belgium	23/29	38.8	Tangni et al., 2003
Apple juice <sup>b</sup>	Belgium	12/14	10.6	Tangni et al., 2003
Apple cider <sup>a</sup>	Belgium	2/5	2.8	Tangni et al., 2003
Apple cider <sup>b</sup>	Belgium	1/2	6.1	Tangni et al., 2003
Apple juice, industrial clear	Belgium	18/21	24.2	Tangni et al., 2003
Apple juice handicraft clear	Belgium	3/4	38.8	Tangni et al., 2003
Apple juice, industrial cloudy	Belgium	8/11	19.8	Tangni et al., 2003
Apple juice handicraft cloudy	Belgium	6/7	26.1	Tangni et al., 2003
Apple juice, conventional	Belgium	11/93	11.6	Baert et al., 2005
Apple juice, organic	Belgium	8/64	122.6	Baert et al., 2005
Apple juice, handicraft	Belgium	3/20	13.6	Baert et al., 2005
Apple juice, conventional <sup>a</sup>	Italy	16/33	53.4	Piemontese et al., 2005
Apple juice, organic	Italy	12/24	69.3	Piemontese et al., 2005
Pear juice, conventional	Italy	1/7	1.1	Piemontese et al., 2005
Pear juice, organic	Italy	4/8	61	Piemontese et al., 2005
Apple juice	Iran	14/42	285.3	Cheraghali et al., 2005
Apple juice concentrates	Iran	13/23	148.8	Cheraghali et al., 2005
Apple juice <sup>b</sup>	Japan	3/143	10	Watanabe and Shimizu, 2005
Apple juice <sup>c</sup>	Japan	6/45	15	Watanabe and Shimizu, 2005
Apple juice <sup>d</sup>	Japan	5/5	9	Watanabe and Shimizu, 2005
Apple juice	Brazil	0/19	—	Iha and Sabino, 2006
Apple vinegar, conventional	Italy	0/2	—	Piemontese et al., 2005
Apple vinegar, organic	Italy	2/6	4.2	Piemontese et al., 2005

<sup>a</sup> Includes one sample of mixture with milk<sup>b</sup> From domestic fruit<sup>c</sup> From imported fruit<sup>d</sup> From domestic fruit with imported concentrate

#### 7.4.6. APPROACHES TO ELIMINATION OF PATULIN FROM FRUIT JUICES

The elimination of patulin production by *Penicillium expansum* or other toxigenic penicillia is critical in maintaining the safety of apple juice, since patulin generally survives the pasteurization process (Tsao and Zhou, 2000). Furthermore, low-temperature storage is not effective in suppressing patulin production, since it may occur at 0°C.

Effort has been dedicated to studying the factors influencing patulin production in fruit juice, and using the knowledge gained in choosing methods for reducing patulin accumulation. These factors include farming practices, such as preharvest fungicidal application, sanitation procedures, and harvesting methods, which should be improved and selected so as to prevent injuries that facilitate fungal penetration into the fruit (Barkai-Golan, 2001). These factors may also include various pre-pressing treatments which will contribute to improved food quality, such as removal of decayed fruit, trimming of moldy portions of the apple, and washing procedures (Jackson et al., 2003). The use of alcoholic fermentation or initial processing that will lead to decomposition of patulin (Stinson et al., 1978), or several postpressing treatments (Kadakal and Nas, 2002), are other approaches to elimination of patulin accumulation. Specific chapters in the present book are dedicated to these subjects.

### 7.5. OTHER *PENICILLIUM* MYCOTOXINS

#### 7.5.1. OCHRATOXIN A

Ochratoxins are a group of closely related derivatives of isocoumarin, linked to L-β-phenylalanine; ochratoxin A (C<sub>20</sub>H<sub>18</sub>O<sub>6</sub>NCl) is the most abundantly produced and the most toxic (Abramson, 1997).

Ochratoxin A is a potent nephrotoxin to many animals and has been associated with the human kidney disease known as Balkan Endemic Nephropathy. It has also exhibited teratogenic, immunotoxic and carcinogenic effects (IARC, 1993; Petzinger and Ziegler, 2000). It is a widespread contaminant in human food, associated with dietary sources that include cereals, legumes, nuts, coffee and cocoa beans, as well as grapes, and wines and other grape-derived products.

The taxonomic status of ochratoxin-A-producing penicillia has changed often during the past century. For years these penicillia were designated as *P. viridicatum* (Larsen et al., 2001), but later studies incorporated ochratoxin-producing penicillia into the species *P. verrucosum* (Pitt, 1987; Larsen et al., 2001). Several studies reported on the production of ochratoxin A by *P. verrucosum* and *P. nordicum* in temperate climates, and by a number of *Aspergillus* species in warmer and tropical areas (Abarca et al., 1994).

*Penicillium verrucosum* and *A. ochraceus* were considered to be the main ochratoxin producers in cereals, and were initially believed to be responsible for producing it in grapes (Ospital et al., 1998). However, studies of grapes of various origins discredited this belief. In a survey of grape samples collected from Argentina and Brazil, da Rocha et al. (2002) found that incidence of *A. ochraceus* was very low and *P. verrucosum* was not detected at all. Similar results were recorded in France: investigating 11 samples of grapes and musts, Sage et al. (2002) found that they were all contaminated by toxigenic strains of *A. carbonarius* and non-toxicogenic strains of *P. chrysogenum*, whereas *A. ochraceus* and *P. verrucosum* were absent.

Battilani and Pietri (2002) found that 95 percent of the fungal strains present on grapes in Italian vineyards belonged to the genus *Aspergillus*, but they also identified *P. pinophilum* among the ochratoxigenic fungi. This species, however, elicited no symptoms on the fruit and, in contrast to the frequently isolated *A. carbonarius*, which was very invasive and penetrated the berries, *P. pinophilum* did not grow within the fruit.

Most of the studies on ochratoxin A contamination in grapes and wines are associated with ochratoxigenic aspergilli, especially *A. carbonarius*. Although *Penicillium* species are most common in harvested and stored grapes, the role of ochratoxigenic penicillia in these products is not sufficiently clear.

### 7.5.2. CITRININ

Citrinin ( $C_{13}H_{14}O_5$ ) is a secondary metabolite, produced mainly by *Penicillium* and *Aspergillus* species. Among the citrinin-producing penicillia we find *P. citrinum*, *P. viridicatum*, *P. expansum*, *P. lividum*, *P. fellutanum*, *P. implicatum*, *P. jensenii*, *P. canescens*, *P. purpurescens*, *P. roqueforti*, *P. thomii*, *P. verrucosum* and other species (Logrieco et al., 2003; Andersen and Frisvad, 2004; Kokkonen et al., 2005). Among these, *P. citrinum*, *P. viridicatum* and *P. expansum* are known postharvest pathogens of fruits and vegetables (Barkai-Golan, 2001).

Of 260 isolates of *P. expansum*, Andersen and Frisvad (2004) reported that 85 percent were citrinin producers, which produced their maximum amounts on yeast-extract sucrose (YES) agar. In a study on toxigenic *Penicillium* species, Kokkonen et al. (2005) found that *P. verrucosum* isolates could produce only citrinin on YES agar, and both citrinin and ochratoxin A on bread analog medium.

Citrinin is an unstable compound: Patterson and Damoglou (1987) monitored its production by *P. viridicatum* in a liquid culture, and found that intracellular factors released by the fungus were responsible for its instability. The major products that resulted from the breakdown of citrinin were dihydrocitrinone and ochratoxin A.

Citrinin is a strong nephrotoxin (Cheeke and Schull, 1985), and it exhibited teratogenic effects on chickens (Ciegler et al., 1977). The renal system of humans was found to be affected and the mitochondrial respiratory chain was identified as a possible sensitive target for this mycotoxin (Ammar et al., 2000). A few studies have also addressed its potential for immunotoxicity (Sharma, 1993).

The production of citrinin by *P. citrinum* gained wide attention because of its involvement in the 'yellow rice' syndrome; it was frequently found in copious quantities, producing its yellow mycotoxin (Saito et al., 1971). Harwig et al. (1979) reported citrinin accumulation in ripe tomatoes infected by *P. expansum*, and Viñas et al. (1993) isolated citrinin-producing strains of *P. expansum* from infected apples in packing houses in Spain. All these citrinin-producing strains were found to produce bacterial metabolites, active against *Bacillus cereus* and *B. subtilis* (Viñas et al., 1993). On the other hand, because of its instability in apple juice, citrinin is not likely to occur in this product (Scott, 2004).

#### 7.5.2.1. Co-occurrence of citrinin and patulin

*Penicillium expansum* strains, capable of synthesizing both patulin and citrinin were isolated from natural rots of apple by Harwig et al. (1973). Since both of these mycotoxins are teratogenic to chicken embryos, their simultaneous administration in various ratios elicited an additive effect from patulin plus citrinin. Examining the toxin-producing capabilities of various strains of *P. expansum* and *P. crustosum*, Ciegler et al. (1977) found that both species could synthesize patulin, whereas only cultures of *P. expansum* isolated from apples produced both patulin and citrinin.

The co-production of patulin and citrinin was studied in 351 samples of apples of various varieties with small rotten areas, collected randomly from markets and supermarkets in Portugal (Martins et al., 2002). No correlation was found between the concentration of patulin or citrinin and the percentage of the whole apple occupied by the rotten areas. The percentage of the samples contaminated with patulin alone was higher (68.6 percent) than that with citrinin alone (3.9 percent), and 19.6 percent of the samples were simultaneously contaminated with both patulin and citrinin. However, different levels of mycotoxins were found in the various apple varieties. The highest level of patulin (80.5 mg/kg) was recorded in cv. Richard, but the citrinin level in this variety was the lowest (0.32 mg/kg); the lowest levels of patulin (3.05–5.37 mg/kg) were found in cvs Rome Beauty, Red Delicious, Golden Delicious and Reineta, and the citrinin level in all these fruits ranged from 0.32 to 0.92 mg/kg. The results indicated that there is a possible risk of human exposure to patulin through the consumption of apple juices and other products derived from apples with small rotten areas (Martins et al., 2002).

Several strains of *P. chrysogenum* and *P. oxalicum* found by Youssef et al. (2000) in dried raisins were reported to be producers of both citrinin and penicillic acid.

#### 7.5.3. PENICILLIC ACID

Penicillic acid ( $C_8H_{10}O_4$ ) was originally isolated from *P. puberulum*, a species which was later identified as *P. verrucosum* var. *cyclopium* by Samson et al.

(1976). This mycotoxin can, however, be produced by various *Penicillium* and *Aspergillus* species. Among the mycotoxigenic penicillia we find *P. cyclopium*, *P. viridicatum* and *P. chrysogenum*, which are included in the list of postharvest pathogens of fruits (Barkai-Golan, 1974) and all of which are producers of penicillic acid.

Penicillic acid is cytotoxic to plant and animal cells and has also been reported as genotoxic to microorganisms (Logrieco et al., 2003).

Although penicillic acid is produced by various penicillia growing on food-stuffs, reports of this toxin in foods are rare, probably as a result of its inherent instability. Similarly to patulin, the unsaturated lactone system reacts readily with sulfhydryl compounds, such as glutathione and cysteine, and is rapidly converted to other products, thereby losing its biological activity (Ciegler et al., 1977).

Several of the *Penicillium* species isolated from wine grapes, including *P. aurantiogriseum*, *P. chrysogenum* and *P. thomii* (Serra et al., 2005), are known to produce penicillic acid and can, therefore, be regarded as potential producers of the mycotoxin in the fruit.

#### 7.5.4. CYCLOPIAZONIC ACID

Cyclopiazonic acid ( $C_{20}H_{20}N_2O_3$ ) is a toxic indole tetramic acid which has been suggested to derive from triptophan (Holzapfel, 1980), and enzymes that catalyze important steps in its biosynthesis have been isolated from *P. cyclopium* (Betina, 1989). Cyclopiazonic acid was subsequently found to be produced by several *Penicillium* and *Aspergillus* species. The *Penicillium* species involved include *P. cyclopium*, *P. puberulum*, *P. viridicatum*, *P. chrysogenum*, *P. crustosum*, *P. patulum*, *P. griseofulvum*, *P. commune*, *P. camembert* and *P. palitans* (Cole, 1984; Frisvad et al., 2004). The first three species are typical postharvest penicillia which naturally infect a variety of harvested fruits (Barkai-Golan, 1974).

Cyclopiazonic acid causes convulsions, degenerative changes and necrosis in the liver, spleen, pancreas, kidney, and other organs of many animals. This mycotoxin has been detected in kodo millet seeds in India, where it was implicated in the human disease 'Kodua Poisoning', and it has been added to the category of potentially serious mycotoxins (Cole, 1984). Cyclopiazonic acid was recorded in six of 22 samples of tomato pulp and two of 22 samples of tomato purée at levels up to 178 and up to 117 ng/g, respectively. Co-occurrence of cyclopiazonic acid and tenuazonic acid was recorded in two samples of tomato purée, and in one sample of tomato pulp (da Motta and Soares, 2001).

Of the many penicillia identified on wine grapes (Serra et al., 2005) several species known to produce cyclopiazonic acid, such as *P. expansum*, *P. chrysogenum*, *P. crustosum* and *P. griseofulvum* can be regarded as potential producers of this mycotoxin.



### 7.5.5. PENITREM A

Penitrems are a group of six related tremorgenic mycotoxins produced by *P. crustosum* (de Jesus et al., 1983), of which penitrem A ( $C_{37}H_{44}NO_6Cl$ ) is the most toxic and the most studied member (Scott, 2004). The fungus can be isolated from melons, in which it causes blue mold rot during storage (Snowdon, 1992). Penitrem A was also produced by a pure culture of *P. expansum* (Bridge et al., 1989), and by *P. carneum* and other penicillia (Frisvad et al., 2004). At low doses it was found to cause tremors in several animals. Dogs were reported to be victims of poisoning via consumption of moldy foods, such as walnuts contaminated with *P. crustosum* (Hocking et al., 1988).

### 7.5.6. CHAETOGLOBOSINS, COMMUNESINS AND OTHER SECONDARY METABOLITES

Examining the ability of 260 isolates of *P. expansum*, obtained from various international culture collections, to produce mycotoxins Andersen et al. (2004) found that none of the isolates produced ochratoxin A or penitrem A, whereas they consistently produced the metabolites chaetoglobosin A and communesin B. Both patulin and roquefortine C were produced by 98 percent of the isolates, and expansolides A and B and citrinin were detected in 91 and 85 percent of the isolates, respectively. Chaetoglobosins, communesins and roquefortine C were originally isolated from apple, pear, quince, apricot, prune, cherry, blackcurrant, fruit juices, strawberry jams and walnuts, as well as from beet roots, brussel sprouts, carrots, licorice roots and tomatoes, and both chaetoglobosins and communesin B were detected in a moldy potato pulp, which caused toxic effects in cows, which were expressed by food refusal (Andersen et al., 2004). Studies by Larsen et al. (1998) showed that cherry juice artificially inoculated with *P. expansum* contained patulin, communesins, chaetoglobosins and expansolides, whereas inoculated blackcurrant juice contained patulin and chaetoglobosin.

The fact that several toxic metabolites may all be found in cherry juice suggested that toxicity to the potential consumer may be increased by possible synergism between the metabolites (Andersen et al., 2004).

Analysis of apple fruit and various juice samples naturally infected by *P. expansum* showed that whereas the whole apple fruit contained patulin, chaetoglobosins A and C, communesin B and roquefortine C, the apple pulp contained only patulin; moldy cherry juice contained chaetoglobosins A and C, communesin B, and roquefortine C; and moldy gooseberry juice contained only chaetoglobosin A and communisin B. Of a special interest is the fact that in a moldy apple that was infected with *P. expansum* while still on the tree only chaetoglobosin A was detected (Andersen et al., 2004).

These results show that a patulin-negative sample does not always indicate that the sample is free of metabolites, and it appears that chaetoglobosin A may

be a better indicator than patulin for the presence of *P. expansum* in the fruit (Andersen et al., 2004). It was also shown that when *P. expansum*, the common pathogen of apples and other deciduous fruits, attacks its host fruit it may produce several toxic metabolites other than patulin during its pathogenesis stages. Andersen et al. (2004) suggested that attention be given to the possible co-occurrence of various *P. expansum* metabolites and products. Apart from *P. expansum*, another *Penicillium* species, *P. discolor*, is known to produce chaetoglobosins (Frisvad et al., 1997).

Chaetoglobosins have been reported to be orally toxic to 2-day-old cockerels (Springer et al., 1976), toxic to embryonic chickens (Veselý et al., 1995), and toxic and teratogenic to mice (Sekita et al., 1982). Communesin B, isolated from a marine alga, was reported to be cytotoxic to lymphocytic leukemia cells (Numata et al., 1993).

Several metabolites of other *Penicillium* species were isolated from moldy tomatoes. They included janthitrems, paspalinine, paxilline and 3-O acetoxypaxilline, all produced by *P. tularense*, and verrucolone produced by *P. olsonii* (Andersen and Frisvad, 2004). The tremorgenic metabolite, paspalinine was found to act on mice, cockerels and domestic animals after oral administration (Gallagher et al., 1980), and paxilline exhibited strong tremorgenic effects on the smooth muscles of several animals (McLeay et al., 1999). Several studies have demonstrated the tremorgenic effects of janthitrems (Cole and Schweikert, 2003).

## 7.6. FUTURE TRENDS

*Penicillium expansum*, the causal agent of the blue mold rot in apples and other deciduous fruits, is a major concern for human health because of the production of patulin during pathogenesis and the subsequent consumption of this mycotoxin in contaminated fruit products. Control of patulin by the processors of fruits and their products will be the essential step toward protecting consumers from potential adverse effects of long-term exposure to this mycotoxin in apple juices, and toward avoiding the exposure of infants to patulin in fruit-based baby products. The recording, in several studies, of higher levels of patulin in organic than in conventional apple products raised the need for stricter control, prior to processing, of fruits grown under organic conditions.

In light of the finding that absence of patulin from a sample of apple products does not always indicate the absence of toxic metabolites, and of the suggestion that chaetoglobosin may be a better indicator than patulin for the presence of *P. expansum*, it was proposed that products potentially contaminated with *P. expansum* should ideally be tested for both chaetoglobosin A and patulin.

The fact that several mycotoxins and other secondary metabolites have been detected together in fruits and fruit-based products should lead to studies of the combined effects of these toxic metabolites.

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# *Alternaria* Mycotoxins

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## ABSTRACT

*Alternaria* species are among the most common postharvest pathogens of fruits and vegetables. During pathogenesis several species are capable of producing several mycotoxins that elicit adverse effects in humans and animals. These mycotoxins include tenuazonic acid, alternariol, alternariol methyl ether, alternuene and altertoxins. Although *A. alternata* has been regarded as the major mycotoxin-producing species, other species such as *A. citri*, *A. solani*, *A. longipes*, and the *A. tenuissima*, *A. arborescens* and *A. infectoria* species-groups may also produce the characteristic *Alternaria* mycotoxins.

*Alternaria* toxigenicity in fruits may vary not only with the fungal species or strain, but also with the host fruit species or cultivar and with the conditions under which the toxin is synthesized. The production of *Alternaria* mycotoxins under natural infection conditions or following inoculation has been observed in a variety of fruits and vegetables, including tomatoes, peppers, melons, apples, blueberries, raspberries, grapes, dried vine fruits, oranges, lemons, mandarins, pecans and olives.

*Alternaria* mycotoxins transferred from a rotten part of the fruit into the surrounding sound tissue may result in the presence of mycotoxins in fruit tissue, even in the absence of fungal mycelium.

The natural occurrence of alternariol at ng/ml levels or of alternariol methyl ether at sub-ng/ml levels, in processed tomato products, in apple juice, apple juice concentrate, grape juice and red wine, raspberry juice, and in cranberry and prune nectars, as well as in olive oil, has been reported.

## 8.1. INTRODUCTION

Many species of the genus *Alternaria* are widely distributed in the soil as normal components of its microflora, and they also occur ubiquitously in the air, as a most important part of the “air spora” worldwide (Gregory, 1973; Barkai-Golan et al., 1977). *Alternaria* species naturally contaminate the aerial parts of plants, and are easily isolated from decaying matter. Various species are plant pathogens that cause damage in the field (Rotem, 1994); others are postharvest pathogens of a wide range of fruits and vegetables (Barkai-Golan, 2001, 2002).

The taxonomy of *Alternaria* has been extensively discussed (Neergaard, 1945; Ellis, 1971, 1976; Kwasna, 1992; Simmons, 1992; Rotem, 1994). The genus comprises more than 100 species, and there is much nomenclature confusion (Kwasna, 1992; Rotem, 1994). Conidial size and shape have been the major criteria used for the identification of *Alternaria* species (Rotem, 1994), but the importance of the three-dimensional sporulation pattern has also been emphasized (Roberts et al., 2000). In addition to the traditional morphological and physiological characteristics, it was suggested that the profile of mycotoxins and other metabolites produced by *Alternaria* would offer advantages in the identification of *Alternaria* species and species-groups (Andersen and Thrane, 1996).

*Alternaria alternata* (Fr.) Keissler, which is the most common species recorded in agricultural commodities, is generally referred to as a collective group of fungi, or an unresolved species-group; it was previously known as *A. tenuis* Nees *auct.* The species is regarded as a saprophyte in food and feed products, but is recognized as a common pathogen of harvested fruits and vegetables, capable of growing during cold storage and transport, and causing postharvest economic losses (Barkai-Golan, 2001).

In addition to spoiling fruits and vegetables through disease formation, *Alternaria* species are also capable, during their pathogenesis stages, of producing a range of toxic metabolites, many of which have not yet been evaluated (Panigrahi, 1997). These metabolites apparently play no role in the primary metabolism of the fungus, and are regarded as secondary metabolites. Most of these secondary metabolites are phytotoxins that play an important role in fungal pathogenicity and are deleterious to crops, but several of them also act as mycotoxins that are harmful to humans and animals. Tenuazonic acid, alternariol, alternariol methyl ether, altenuen and altertoxin-I are the main *Alternaria* mycotoxins produced in fruits and vegetables, following fungal infection.

Although *A. alternata* has been regarded as the major mycotoxin-producing species, several other *Alternaria* species are known to produce these mycotoxins, and increased attention has recently been drawn towards *A. tenuissima* isolates as important producers of *Alternaria* mycotoxins.

Since *Alternaria* mycotoxins may be produced naturally in a variety of infected fruits and vegetables intended for human consumption and since *Alternaria* infection may occur under refrigeration, these mycotoxins may be considered as toxic contaminants in our everyday food.

## 8.2. SIGNIFICANCE OF ALTERNARIA SPECIES AS PATHOGENS OF FRUITS AND VEGETABLES

The optimal temperature range for *Alternaria* growth (22–28°C) enables the fungus to grow well at room temperature in various climate regions. However, *Alternaria* is also capable of growing at low temperatures, and its minimal developmental temperature is –3°C (Sommer, 1985). That is why the fungus

is often involved in spoilage during refrigerated storage, and continues to develop in cabbage, celery and other vegetables stored at 0°C, or in certain apple cultivars stored at 0°C or below. Tomato fruits stored at temperatures lower than 8–12°C are extremely sensitive to *A. alternata*, even before the appearance of chilling injury symptoms. Similarly, sweet peppers are injured below 7°C and become susceptible to *Alternaria* decay, and sweet potatoes are predisposed to *Alternaria* when stored below 10°C (Barkai-Golan, 2001).

The fact that *Alternaria* is capable of growing at low temperatures may also lead to its occurrence on fruits and vegetables subjected to cold stress and thereby sensitive to disease initiation.

*Alternaria alternata*, the most common species that attacks harvested produce, is generally unable to penetrate the sound host directly through the plant cuticle or epidermis; it requires suitable openings, such as the calyx scar or wounded tissue, for invasion, and such opportunities occur frequently during harvesting and handling. This species is a very common pathogen of the solanaceous fruits, including tomatoes, peppers and eggplants (Barkai-Golan, 2002), in which infection is often initiated at the calyx scar (Dennis, 1983; Fallik et al., 1994). Incipient infections, which may appear at the margin of the stem scar, remain quiescent unless the fruits are subjected to weakening conditions or situations, such as chilling injury, sunscald, over-maturity and prolonged storage.

In view of the high incidence of *A. alternata* in harvested tomatoes, it is important to emphasize the high level of genetic diversity found among isolates collected from typical tomato black lesions (Morris et al., 2000). The isolation and characterization of five microsatellite markers for studying *A. alternata* has recently been described (Tran-Dinh and Hocking, 2006): marker polymorphism was screened in 64 isolates of *A. alternata*, and was considered to be potentially useful in the study of relationships and population genetics amongst isolates of this species.

In addition to *A. alternata*, other *Alternaria* species were found to be associated with postharvest spoilage of tomatoes: *A. tenuissima* (Logrieco et al., 2003; Andersen and Frisvad, 2004; Pose et al., 2004), *A. arborescens* (Filtenborg et al., 2002), and *A. longipes* (Pose et al., 2004). Furthermore, in a study by Andersen and Frisvad (2004) in Denmark, members of the *A. tenuissima* species-group were found to be the most dominant fungi on moldy tomatoes.

*Alternaria alternata* is a common postharvest pathogen of various cucurbits, in which the common points of penetration are the cut stem or injuries to the rind. Cucumbers, squashes and melons are frequently infected after being weakened by sunscald, chilling injury or prolonged storage (Snowdon, 1992).

*Alternaria radicina* and *A. dauci* may attack carrot roots, especially those that display cracks or splits (Snowdon, 1992). During harvesting and handling, potato tubers contaminated with *A. alternata* spores are subject to invasion through injuries that occur especially during mechanical harvesting (Droby et al., 1984).

*Alternaria alternata* is a common postharvest pathogen of pome and stone fruits. Fungal spores, which are generally present on the fruit surface, preferably enter through wounds formed during harvesting and handling (Barkai-Golan, 2001). In several pome fruits the fungus can also penetrate the fruit via open calyces, and in cherries penetration can take place through splits formed in the skin when the fruit is still on the tree (Snowdon, 1990). Although *Alternaria* core rot in apples has frequently been associated with *A. alternata*, recent studies in South Africa indicated that *Alternaria* isolates classified as *A. infectoria*, *A. arborescens* and *A. tenuissima* species-groups were all associated with this disease (Serdani et al., 2002). Classification of *A. infectoria* species-groups based on sporulation patterns, and cultural, biochemical and genetic data enabled them easily to be differentiated from *A. arborescens* and *A. tenuissima* species-groups, since the last two species-groups are typically clustered together. This study also showed that the major pathogens involved in core rot disease of “Top Red” apples belonged to the *A. tenuissima* species-group (Serdani et al., 2002).

In persimmon fruits, *A. alternata* penetration frequently occurs via small cracks formed beneath the calyx as the fruit approaches maturity. Direct penetration of developing fruits can occur during the growth period, but the infection remains quiescent and resumes its activity only after harvest, when the fruit ripens (Prusky et al., 1981). In kiwifruit this weak pathogen may grow on the fruit surface but has not been considered a primary pathogen (Eckert and Ogawa, 1988).

*Alternaria alternata* is an important postharvest pathogen of grapes, in which infection is often initiated at the stem-end (Hewitt, 1974). It is a common pathogen in wine grapes and has been recorded in 80 percent of the fruits collected in Argentina (Magnoli et al., 2003). The fungus is of minor importance in strawberries and raspberries, but may be one of the main pathogens of blueberries and gooseberries (Dennis, 1983; Wright et al., 2004).

*Alternaria alternata* is a common pathogen of mango, in which penetration occurs via lenticels, but the infection remains quiescent until the onset of ripening, after harvest (Prusky et al., 1983). The fungus can also attack papaya fruits, especially after prolonged storage (Barkai-Golan, 2001).

Postharvest black rot of citrus fruit was found to be associated with peel wounds; it is caused by phylogenetically distinct lineages of *A. alternata*, and has been mainly associated with isolates capable of producing endopolygalacturonase (Timmer et al., 2003; Peever et al., 2005). *A. citri*, which is one of the main stem-end pathogens of citrus fruits, is frequently found at the stem-end or underneath the bottom of the fruit. However, infection can occur only if the fruit has been injured or physiologically weakened by unfavorable growing conditions (Eckert and Eaks, 1989).

*Alternaria alternata* often affects olives when the fruits remain on the soil for a long time after ripening (Visconti et al., 1986). Surface damage caused by low temperatures and other unfavorable conditions is a major precondition for fungal penetration into the fruit pulp, where subsequent mycelium development takes place (Visconti et al., 1986; Logrieco et al., 2003).

### 8.3. ALTERNARIA MYCOTOXINS

#### 8.3.1. THE MAJOR MYCOTOXINS AND THEIR PRODUCERS

More than 120 secondary metabolites of *Alternaria* are known, although a large proportion of them have been reported as phytotoxic compounds, and only about a quarter act as mycotoxins to humans and animals (Panigrahi, 1997).

*Alternaria alternata*, which is the most common *Alternaria* species in harvested fruits and vegetables, is probably the most important mycotoxin-producing species. It produces a number of mycotoxins, belonging to different structural classes, of which the five major compounds are alternariol ( $C_{14}H_{10}O_5$ ), alternariol methyl ether ( $C_{15}H_{12}O_5$ ) and altenuene ( $C_{15}H_{16}O_6$ ), which are benzopyrone derivatives; tenuazonic acid ( $C_{10}H_{15}NO_3$ ), which is a tetramic acid derivative; and altertoxin-I ( $C_{20}H_{16}O_6$ ), a perylene derivative. The ability of *A. alternata* to produce several different mycotoxins may explain the particular interest of mycotoxicologists in this species (Scott, 2004).

However, other *Alternaria* species are also capable of producing several mycotoxins. Thus, alternariol and alternariol monomethyl ether may also be produced by *A. tenuissima*, *A. brassicae*, *A. capsici-annui*, *A. citri*, *A. cucumerina*, *A. dauci*, *A. kikuchiana*, *A. longipes*, *A. porri*, *A. solani* and *A. tomato* (Bottalico and Logrieco, 1998; Andersen and Frisvad, 2004; Pose et al., 2004). Tenuazonic acid, which is produced in culture by numerous strains of *A. alternata*, also may be produced by several other *Alternaria* species, including *A. capsici-annui*, *A. citri*, *A. japonica*, *A. kikuchiana*, *A. longipes*, *A. porri*, *A. radicina*, *A. tenuissima* and *A. tomato* (Bottalico and Logrieco, 1998; Pose et al., 2004). Altertoxin I and the related perylene derivatives, altertoxins II and III, are also produced by *A. tenuissima*, *A. mali*, *A. radicina* and *A. tomato* (Bottalico and Logrieco, 1998).

Analyses of metabolite profiles of 153 *Alternaria* isolates belonging to the *A. infectoria*, *A. arborescens* and *A. tenuissima* species-groups showed that the *A. infectoria* species-group, which is chemically different from the other two species-groups, has only a few metabolites in common with them (Andersen et al., 2002). In contrast, the *A. arborescens* and *A. tenuissima* species-groups shared most of the known metabolites, including alternariols, tenuazonic acid, altenuen and altertoxins (Andersen et al., 2002; Andersen and Thrane, 2006).

Although *A. alternata* has generally been considered a most important pathogen of tomatoes (Barkai-Golan, 2001; Logrieco et al., 2003), a study of mycotoxins in naturally infected tomatoes indicated that all the 22 *Alternaria* isolates originated from same source belonged to presently undescribed taxa in the *A. tenuissima* species-group (Andersen and Frisvad, 2004).

Studying the toxic metabolites of four *A. tenuissima* species-groups isolated from tomato lesions, Andersen and Frisvad (2004) found that all four produced tenuazonic acid, three of them also produced alternariols and three also produced altertoxin I. Another *A. tenuissima* metabolite, altersetin, was



produced by two of the *A. tenuissima* species-groups. The metabolite tentoxin, which is regarded as a phytotoxin (Ramm et al., 1994), has also been isolated from tomato lesions (Andersen and Frisvad, 2004). Other fungal species isolated from moldy tomatoes were *Stemphylium* cf. *lycopersici*, the producer of stemphols, and *Stemphylium eturmiunum*, the producer of the metabolites infectopyrone and macrosporin (Andersen and Frisvad, 2004).

Newly harvested, undamaged apples were usually free of fungal infection. Analysis of the mycoflora of infected apples showed that many of the fungal species present in the mature fruits were already present in the apple blossom, and later colonized the young immature fruits (Andersen and Thrane, 2006).

The dominant *Alternaria* isolates on apples were those belonging to the *A. tenuissima* species-group followed by isolates belonging to *A. arborescens* species-group. On cherries the dominant isolates were those belonging to *A. arborescens* species-group followed by *A. tenuissima* isolates (Andersen and Thrane, 2006). In fact, the taxonomic literature concerning *Alternaria* is under revision, indicating that *A. alternata* is not as common as presumed (Samson et al., 2002).

### 8.3.2. TOXIC EFFECTS OF *ALTERNARIA* MYCOTOXINS

Toxic effects of *A. alternata* mycotoxins on the liver and kidney of rats feeded by the fungus for 28 days have been described (Combina et al., 1999). Alternariol monomethyl ether produced by *A. solani* was found to be toxic to hamsters when applied intraperitoneally at 200 mg/kg bw, whereas no toxic effects were observed at a dose level of 50 or 100 mg/kg bw (Pollock et al., 1982). Both alternariol and alternariol monomethyl ether, which are frequently found in combination, were found to be mutagenic (Schrader et al., 2001). The mutagenicity of altertoxins I, II and III to *Salmonella typhimurium* was exhibited by Stack and Prival (1986). Of the three mycotoxins, altertoxin III was found to be the most mutagenic compound.

Of particular health concern is the association found between *A. alternata* contamination in cereal grains and the high levels of human esophageal cancer in China (Liu et al., 1991); the disclosure that contamination of food with alternariol-producing strains of *A. alternata* might be associated with esophageal cancer raised concern about exposure to low levels of alternariol (Brugger et al., 2006; Lehmann et al., 2006). Lehmann et al. (2006) have recently reported on the estrogenic potential of alternariol, its inhibitory effects on cell proliferation, and its genotoxic effect in cultured mammalian cells. Studying the mutagenicity of alternariol in cultured mammalian cells, Brugger et al. (2006) suggested that this may have a bearing on its carcinogenicity.

Tenuazonic acid has been considered a most important toxic compound produced by *Alternaria*, in light of the positive correlation between the in vitro production of the mycotoxin and the toxicity of cultures to laboratory animals (Viñas et al., 1992). Tenuazonic acid is toxic to several animal species, including dogs, in which it causes hemorrhages in several

organs at daily doses of 10 mg/kg bw, and chickens, in which it causes sub-acute toxicity when added to their feed at 10 mg/kg bw (Scott, 2004). Pre-cancerous changes in the esophageal mucosa of mice fed with tenuazonic acid at 25 mg/kg bw per day for 10 months were reported by Yekeler et al. (2001). The possible involvement of tenuazonic acid in the etiology of onyalay, a human hematological disorder occurring in Africa, has been suggested (Logrieco et al., 2003).

Tenuazonic acid is also toxic to a wide range of plants, fungi, bacteria and viruses (Logrieco et al., 2003) and was reported as a phytotoxin produced by various *Alternaria* species (Harvan and Pero, 1976).

Altertoxin I and related compounds may cause acute toxicity in mice, and were reported to be more potent mutagens than alternariol and alternariol monomethyl ether (Chelkowski and Visconti, 1992; Schrader et al., 2001). In fact, the interest in these toxins is focused on their mutagenic activity.

### 8.3.3. FACTORS AFFECTING MYCOTOXIN PRODUCTION

Mycotoxin production depends on the fungal strain, the substrate on which it grows and the environmental growth conditions. Diverse isolates of *Alternaria* spp., most of which belonged to *A. alternata*, were found to produce alternariol and alternariol methyl ether in complex liquid media and in solid rice medium (Mass et al., 1981). Although toxin production varied with the isolate, the production by each isolate depended upon the culture medium. The highest yields of toxins were obtained from rice cultures, in which toxin levels varied from 21 to 53 µg/g for alternariol and from 70 to 417 µg/g for alternariol methyl ether. Culturing *A. alternata* in vitro, in synthetic or semisynthetic liquid media, Wei and Swartz (1985) found that alternariol, alternariol methyl ether and altenuene were synthesized during the later stages of growth, and that higher toxin levels were produced in a semisynthetic medium. An association was found between the differing activity levels of the fungus on the two media and the pH of the media during fungal growth: a decrease from 4.0 to 2.1 was recorded on the synthetic medium, and an increase from 5.1 to 6.8 on the semisynthetic medium (Wei and Swartz, 1985). In a given semisynthetic medium, reductions in the carbon source (glucose) levels within the medium, which changed the C/N ratio from 2:1 to 6:1, were found to greatly increase both fungal growth and toxin production (Wei and Swartz, 1985). For a given *A. alternata* isolate, different optimal temperatures were recorded for the production of different mycotoxins in a synthetic medium: 28°C for alternariol and alternariol methyl ether, 21°C for tenuazonic acid and 14°C for altertoxin-I and altertoxin-II. Noticeable levels of both growth and toxin production occurred at 7°C, but the levels dropped at 35°C (Hasan, 1995).

The effect of temperature on mycotoxin production was also noticed in infected fruits. Since *A. alternata* is particularly well known as a tomato pathogen, it was not surprising to find that fungal growth and toxin production in this fruit occurred at a wide range of incubation temperatures: from

4°C to 25°C (Özcelik et al., 1990). Although the fungus grew and produced toxins more rapidly at 25°C than at lower temperatures, concentrations of alternariol up to 1206 µg/g and of alternariol methyl ether up to 637 µg/g were recorded in tomatoes stored at 15°C for 4 weeks (Özcelik et al., 1990).

Evaluating the importance of *A. alternata* mycotoxins in apples at different temperatures, Viñas et al. (1992) found that 47 and 41 percent of the fruits held at 25°C produced alternariol and alternariol methyl ether, respectively, and more than 38 percent of them produced both toxins simultaneously. However, *A. alternata* strains were also capable of growing and of producing mycotoxins in apples held at 2°C, although lower percentages of strains produced these toxins: 17.6 percent produced alternariol, 5.9 percent produced alternariol methyl ether and 5.9 percent produced both.

Decreases in the concentrations of alternariol and alternariol methyl ether in *A. alternata*-infected tomatoes were recorded during the course of storage. It was suggested that some degradation of toxins may occur after prolonged storage, especially at 25°C (Özcelik et al., 1990).

Studies of toxin formation in olives indicated that surface physical damage, which allowed fungal penetration into the fruit pulp and the subsequent mycelial growth and toxin production, was an important factor in influencing toxin production (Visconti et al., 1986).

## 8.4. OCCURRENCE OF *ALTERNARIA* MYCOTOXINS IN FRUITS AND VEGETABLES

The production of *Alternaria* mycotoxins under natural infection conditions or following inoculation has been studied in a variety of fruits and vegetables. Mycotoxin occurrence has been reported in tomatoes (Harwig et al., 1979; Stinson et al., 1980, 1981; Özcelik et al., 1990; HyangBurm and SeungHun, 1995; Scott, 2001; Andersen and Frisvad, 2004); apples (Stinson et al., 1980, 1981; Özcelik et al., 1990; Viñas et al., 1992; Robiglio and Lopez, 1995; Singh and Geeta Sumbaly, 2004); blueberries (Stinson et al., 1980, 1981); grapes and dried vine fruits (Tournas and Stack, 2001; Romero et al., 2005); oranges and lemons (Stinson et al., 1981; Logrieco et al., 1990); mandarins (Logrieco et al., 1990); olives (Visconti et al., 1986) and pecans (Schroeder and Cole, 1977). However, various qualitative and quantitative studies clarified that *Alternaria* toxigenicity in fruits may vary not only with fungal species or strain, but also with the host fruit species or cultivar, and with the conditions under which the mycotoxin may be synthesized in the host fruit.

Studies by Özcelik et al. (1990) indicated that alternariol and alternariol methyl ether were the main mycotoxins formed in tomatoes and apples following inoculation with *A. alternata*; they compared the toxin levels in tomatoes and apples after 3 weeks at 25°C, and recorded maximum alternariol concentrations of 1161 and 372 µg/g, respectively, and maximum alternariol methyl ether concentrations of 323 and 32 µg/g, respectively, under the same conditions (Table 8.1).

TABLE 8.1 *Alternaria* mycotoxins in fruits under natural conditions or following inoculation.

Mycotoxin <sup>a</sup>	Commodity	Maximal toxin levels (µg/g)	References
TA	Tomatoes <sup>i</sup>	106.0	Harwig et al., 1979
AOH		0.0	
AME		0.8	
TA	Tomatoes <sup>i</sup>	1373.0	Stinson et al., 1980
AOH		569.0	
AME		171.0	
ALT		16.0	Stinson et al., 1981
TA	Tomatoes <sup>n</sup>	139.0	
AOH		5.3	
AME		0.8	
ALT		1.1	
TA	Tomatoes <sup>n</sup>	70.0	Stack et al., 1985
TA	Tomatoes <sup>i,b</sup>	0.0	
AOH		1161.0	Özcelik et al., 1990
AME		323.0	
ALT		190.0	Giryn and Szteke, 1995
AOH	Tomatoes <sup>n</sup>	0.4	
AME		0.1	
TA	Tomatoes <sup>n</sup>	7.2	Bottalico and Logrieco, 1998
AOH		1.3	
AME		0.3	Stinson et al., 1981
TA	Apples <sup>n</sup>	0.5	
AOH		58.8	
AME		2.3	Özcelik et al., 1990
ALT		0.5	
TA	Apples <sup>i,b</sup>	0.0	
AOH		372.0	Singh and Geeta Sumbaly, 2004
AME		32.2	
ALT		22.2	
TA	Apples <sup>n</sup>	9.6	Tournas and Stack, 2001
AOH	Apples <sup>i</sup>	5.0	
AME		14.0	
AOH	Grapes <sup>i</sup>	3336.0	Tournas and Stack, 2001
AME		1716.0	
TA	Blueberries <sup>i</sup>	202.5	Stinson et al., 1980
AOH		72.5	
AME		209.0	
ALT		20.6	Stinson et al., 1981
TA	Oranges <sup>i</sup>	61.1	
AOH		41.0	
AME		33.3	
ALT		15.9	
TA	Lemons <sup>i</sup>	48.8	Stinson et al., 1981
AOH		2.8	
AME		4.4	
ALT		0.5	Logrieco et al., 1990
TA	Mandarins (black rot) <sup>n</sup>	87.2	
AOH		5.2	
AME		1.4	Logrieco et al., 1990
TA	Mandarins (gray rot) <sup>n</sup>	173.9	

(Continued)

TABLE 8.1—(Cont'd)

Mycotoxin <sup>a</sup>	Commodity	Maximal toxin levels (µg/g)	References
TA	Olives <sup>n,c</sup>	0.3	Visconti et al., 1986;
AOH		2.3	Bottalico and Logrieco,
AME		2.9	1993
ALT		1.4	
ATX-I		0.2	

<sup>a</sup>ALT – altenuene; AME – alternariol methyl ether; AOH – alternariol; ATX-I – altertoxin-I; TA – tenuazonic acid.

<sup>b</sup>At 25°C.

<sup>c</sup>Badly damaged, weathered or moldy olive sample.

<sup>f</sup>Following inoculation.

<sup>n</sup>Under natural conditions.

In contrast to the findings of Özcelik et al. (1990), who did not detect any tenuazonic acid in *Alternaria*-inoculated tomatoes or apples (Table 8.1), earlier studies by Harwig et al. (1979) in ripe tomatoes inoculated with toxigenic isolates of *A. alternata* found tenuazonic acid at levels up to 106 µg/g, and studies by Stinson et al. (1980, 1981) in naturally infected and in inoculated tomatoes reported on tenuazonic acid at levels up to at 139 and 1373 µg/g fruit tissue, respectively. In fact, these studies found tenuazonic acid to be the major mycotoxin in tomatoes, in spite of the differences between the findings of the three studies. In a survey of moldy tomatoes collected from the processing lines of catsup manufacturers, Stack et al. (1985) found that whereas 69 of 142 samples analyzed were negative for tenuazonic acid, 28 contained up to 1.9 µg/g, and 45 up to 70 µg/g (Table 8.1).

Tenuazonic acid was the major *Alternaria* mycotoxin produced in naturally infected tomatoes collected in southern Italy, with levels up to 7.2 µg/g. Lower levels were recorded for alternariol and alternariol methyl ether (Bottalico and Logrieco, 1998). Altenuen, which, like alternariol and alternariol methyl ether, is also a benzopyrone derivative, was found in smaller amounts in each of the fruits tested, and frequently at trace levels only (Table 8.1).

As a result of inoculation studies, the potential for the occurrence of *Alternaria* mycotoxins in various fruits has been established. Thus, following inoculation of blueberries with *A. alternata* isolated from decayed blueberries, the production of tenuazonic acid (up to 2025 µg/g) was demonstrated (Stinson et al., 1980). The potential for altertoxin-I production in tomatoes was demonstrated, following inoculation with *A. alternata* (Stinson et al., 1980), although no altertoxin-I was recorded in tomatoes under natural infection conditions (Stinson et al., 1981).

The production of tenuazonic acid in oranges and lemons, at levels up to 61.1 and 48.8 µg/g, respectively, was recorded, following inoculation of the fruits with *A. citri*. Levels up to 41 and up to 2.8 µg/g of alternariol and alternariol methyl ether, respectively, were also produced in inoculated oranges and lemons (Stinson et al., 1981). Studying the relative importance of the mycotoxins produced in inoculated oranges and lemons, Stinson et al.

(1981) found similar patterns of production in the two fruits, although the overall mycotoxin contents were higher in oranges than in lemons.

Two different strains of *A. alternata*, which were isolated by Logrieco et al. (1990) from a natural black or gray “heart rot” of mandarins, were found to differ, not only in their pigmentation, but also in their abilities to produce mycotoxins. The black rot samples contained tenuazonic acid, alternariol and alternariol methyl ether, of which tenuazonic acid was the most abundant, whereas tenuazonic acid was the only toxin found in the gray rot sample, at up to 173.9 µg/g (Table 8.1).

Examining the mycotoxin-producing potential of *A. alternata* in “Red Delicious” apples, Singh and Geeta Sumbaly (2004) found that 83 percent of the *A. alternata* isolates produced tenuazonic acid as the main mycotoxin in the inoculated apples, with the highest level being 96 µg/g (Table 8.1). No detectable amounts of alternariol and alt毒素-I were found in the rotten apple tissues or their surroundings.

Working with two toxigenic strains of *A. alternata*, Tournas and Stack (2001) found that the fungus and its mycotoxins were not a major problem in strawberries, because of the presence of fast-growing molds, such as *Rhizopus* and *Botrytis*, which outgrow *Alternaria* and inhibit its growth. The growth of both *Alternaria* strains on apples was limited, even without the presence of fast-growing molds. Both alternariol and alternariol methyl ether were produced, at up to 5 and up to 14 µg/g, respectively, by one strain of *A. alternata* in inoculated ‘Golden Delicious’ and ‘Gala’ apples. Moderate *Alternaria* growth, along with the production of alternariol and alternariol methyl ether, at up to 3336 µg/g and up to 1716 µg/g, respectively, were recorded by Tournas and Stack (2001) in grapes (Table 8.1).

The toxigenic profiles of *A. alternata* and *A. radicina* from carrot were determined by growing the fungi on rice and carrot discs. Most of the *A. alternata* isolates produced tenuazonic acid, alternariol, alternariol methyl ether and alt毒素-I when cultured on rice, but only alternariol and alternariol methyl ether when cultured on carrot discs (Solfrizzo et al., 2005). Although the potential for mycotoxin production by *A. alternata* in carrot has been demonstrated, no *Alternaria* mycotoxins were detected in carrot roots during the production and storage of carrots and their products (Solfrizzo et al., 2005). Another *Alternaria* species, *A. radicina*, which infects carrot roots in the field and postharvest (Snowdon, 1990), is known to produce radicinin and radicnols, which exhibit phytotoxic activity (Solfrizzo et al., 2005). Radicinin, produced by *A. radicina*, was detected at 0.16–13.9 µg/g in three out of 266 carrot samples produced under organic conditions in European locations, whereas *A. alternata* mycotoxins were not found in any of the samples (Solfrizzo et al., 2004).

Evaluation of the potential for mycotoxin production by fungi in dried vine fruits indicated that several *A. alternata* strains were producers of tenuazonic acid, alternariol and alternariol methyl ether (Romero et al., 2005).

The natural occurrence of alternariols was reported in pecans (Schroeder and Cole, 1977). However, their production was limited to discolored pecan

kernels, which are removed from shelled pecans during processing, which led to the suggestion that such pecans would be rejected by buyers of in-shell pecans (Schroeder and Cole, 1977).

The natural occurrence of the major *Alternaria* mycotoxins in olives and related processing products (oil and husks) was first reported by Visconti et al. (1986). None of the undamaged olives properly harvested from the ground in several areas were found to contain mycotoxins, despite the presence of *Alternaria* on the olive surface; of the 13 olive samples collected, 4 were contaminated with 2–4 *Alternaria* mycotoxins. Heavily damaged olives contained considerable amounts of *Alternaria* mycotoxins, the highest levels being 0.26, 2.9, 2.3 and 1.4 µg/g of tenuazonic acid, alternariol methyl ether, alternariol and altenuene, respectively (Table 8.1). Altertoxin-I was not detected in any sample at levels higher than 0.2 µg/g. These studies indicated that toxin production in olives is probably associated with physical damage to the fruit surface, which would allow fungal penetration into the fruit pulp, with subsequent mycelia growth and toxin synthesis.

A large amount of tenuazonic acid was produced when *A. alternata* isolated from olives was grown on rice: the tenuazonic acid content reached up to 980 µg/g and the average exceeded 60 µg/g (Visconti et al., 1986).

The maximal levels of *Alternaria* mycotoxins recorded in fruits under natural conditions or following inoculation are given in Table 8.1.

Various food products may be profusely supplemented with various amounts of fresh or dried fruits and vegetables, which may be contaminated by diverse fungi, and become good substrates for the production of mycotoxins. Such supplements may subsequently cause slow toxicoses (Lugauskas et al., 2005). Food products contaminated by *Alternaria*, *Penicillium*, *Aspergillus*, *Fusarium* and other fungal genera because of supplementation with fruits and vegetables should, therefore, receive particular attention.

## 8.5. TRANSFER OF *ALTERNARIA* MYCOTOXINS IN FRUITS AND THEIR PRESENCE IN FRUIT-DERIVED PRODUCTS

### 8.5.1. MYCOTOXIN TRANSFER

Studying mycotoxin production in moldy core disease in “Red Delicious” apples in storage, Robiglio and Lopez (1995) isolated 11 strains of *A. alternata*, the most common pathogenic cause of this disease. Most of the isolates produced alternariol and alternariol methyl ether in the whole fruits. Studies on the possible transfer of *Alternaria* mycotoxins from the rotten part of an inoculated fruit to the surrounding sound tissues indicated that toxins were not restricted to the rotted area, which was characterized by abundant fungal hyphae; they could also be isolated from the surrounding tissue, although comparative evaluation showed that toxin levels were lower (Robiglio and Lopez, 1995). Furthermore, mycotoxins could be detected in mycelium-free

filtrate derived from liquid cultures of *A. alternata*, as well as in asymptomatic tissues from inoculated fruits. It was, therefore, concluded that the possible presence of *A. alternata* mycotoxins in fruit tissues should be considered, even in the absence of fungal mycelium.

### 8.5.2. ALTERNARIA MYCOTOXINS IN FRUIT JUICES AND OTHER FRUIT PRODUCTS

*Alternaria* mycotoxins have been recorded in various processed fruits, including tomato products (Scott and Kanhere, 1980; Stack et al., 1985; Giryn and Szteke, 1995; Fente et al., 1998; da Motta and Soares, 2001), apple juice (Giryn and Szteke, 1995; Scott et al., 1997; Scott, 2001; Lau et al., 2003), apple juice concentrate (Delgado and Gómez-Cordovés, 1998), grape juice (Lau et al., 2003; Scott et al., 2006), raspberry juice (Giryn and Szteke, 1995; Lau et al., 2003), prune and cranberry nectar (Lau et al., 2003).

The natural occurrence of alternariol and alternariol methyl ether was reported in 50 percent of 32 samples of commercial apple juice in Spain, which were analyzed by Delgado and Gómez-Cordovés (1998). Alternariol levels ranged from 1.35 to 5.42 ng/ml, and alternariol methyl ether was frequently present, but only at low or trace levels, the highest concentration – 1.71 ng/ml – being detected in one sample. Alternariol and, to a lesser extent, alternariol monomethyl ether occurred naturally in apple juice concentrates used in the production of commercial reconstituted apple juices (Delgado and Gómez-Cordovés, 1998).

Confirming the natural occurrence of alternariol in nine apple juice samples and in single samples of some other fruit beverages, including grape juice, raspberry juice, cranberry nectar, prune nectar and red wine, Lau et al. (2003) found that alternariol appeared at levels up to 6 ng/ml, and that the prune nectar contained also alternariol methyl ether at 1.4 ng/ml. Analyzing wines, and grape and cranberry juices for *Alternaria* mycotoxins, Scott et al. (2006) found alternariol at 0.03–5.02 ng/ml in 13 of 17 local Canadian red wines, and at 0.27–19.4 ng/ml in all seven imported red wines; it was usually accompanied by lower concentrations of alternariol methyl ether. White wines contained only sublevels of the two mycotoxins ( $\leq 1.5$  ng/ml), both of which were found to be stable in the wine (Scott and Kanhere, 2001). Sublevels of the two mycotoxins were also reported in 5 of 10 samples of red grape juice, except for one sample that contained alternariol methyl ether at 39 ng/ml, and in one of five samples of cranberry juice. No toxins were recorded in white grape juices.

Since *Alternaria* species are ubiquitous in nature and are very commonly occurring postharvest pathogens of tomatoes, it is no wonder to find *Alternaria* mycotoxins in tomato products (Scott and Kanhere, 1980; Stack et al., 1985; Fente et al., 1998; da Motta and Soares, 2001). Tenuazonic acid was found by Scott and Kanhere (1980) in tomato paste at levels up to 100 ng/g, and alternariol was recorded in tomato paste at levels up to 196 ng/g by Fente



et al. (1998). Tenuazonic acid at 39–111 ng/g and 29–76 ng/g, respectively, was reported by da Motta and Soares (2001) in 7 of 22 tomato pulp samples and in 4 of 22 tomato purée samples.

The possible transfer of *Alternaria* mycotoxins in olive fruits was studied by Visconti et al. (1986) and Bottalico and Logrieco (1993). No mycotoxins were detected in olive oil destined for human consumption or in olive husks collected from oil mills after the first pressing of olives. However, an olive oil sample produced in the laboratory by processing the most contaminated olive sample contained alternariol at 793.6 ng/g and alternariol methyl ether at 285.7 ng/g. The estimated percentages of mycotoxins transferred into the oil were about 4 percent for alternariol methyl ether, 1.8 percent for alternariol

TABLE 8.2 Content of *Alternaria* mycotoxins in fruit juices and other fruit products.

Mycotoxin <sup>a</sup>	Product	Toxin level (maximum) ng/ml or ng/g	References
TA	Tomato paste	100.0	Scott and Kanhere, 1980
TA	Tomato pulp	111.0	Da Motta and Soares, 2001
TA	Tomato purée	76.0	da Motta and Soares, 2001
AOH	Processed apple products	Traces	Giryn and Szteke, 1995
AOH	Apple juice concentrate	5.4	Delgado and Gómez-Cordovés 1998
AME		1.7	
AOH	Apple juice	2.4	Lau et al., 2003
AME		0.4	
AOH	Raspberry juice	0.8	Lau et al., 2003
AME		ND	
AOH	Prune nectar	5.5	Lau et al., 2003
AME		1.4	
AOH	Cranberry juice	ND	Lau et al., 2003
AME		ND	
AOH	Cranberry nectar	5.6	Lau et al., 2003
AME		0.7	
AOH	Grape juice	1.6	Lau et al., 2003
AME		0.2	
AOH	Red grape juice	<1.5	Scott et al., 2006
AME		<1.5 <sup>b</sup>	
AOH	Red wine	1.9	Lau et al., 2003
AME		ND	
AOH	Red wine <sup>c</sup>	5.0	Scott et al., 2006
	Red wine <sup>d</sup>	19.4	
AOH	Olive oil <sup>e</sup>	793.6	Visconti et al., 1986
AME		285.7	

<sup>a</sup>AME – alternariol methyl ether; AOH – alternariol; TA – tenuazonic acid.

<sup>b</sup>With the exception of one sample with 39 ng/ml.

<sup>c</sup>Canadian wine, local.

<sup>d</sup>Canadian wine, imported.

<sup>e</sup>Produced in the laboratory from the most contaminated olive sample.

and 0 percent for altenuene and tenuazonic acid. The *A. alternata* strains isolated from olive oil produced higher levels of mycotoxin when grown on rice medium than on the olive itself.

The incidence and levels of *Alternaria* mycotoxins in fruit juices and other fruit products are given in Table 8.2.

Co-occurrence of tenuazonic acid and cyclopiazonic acid, at 36–117 ng/g, in 2 of 22 samples of tomato purée and at 29–76 ng/g in 6 of 22 samples of tomato pulp was recorded by da Motta and Soares (2001); neither alternariol methyl ether nor alternariol was detected in the various tomato samples. It was suggested that the presence of tenuazonic acid and cyclopiazonic acid in tomato products may indicate the use of decaying tomatoes in the processing procedure.

## 8.6. FUTURE TRENDS

In view of the widespread occurrence of *Alternaria* in harvested fruits and vegetables intended for human consumption, and the high levels of toxicity found in some of the most important fruits for human consumption (such as tomatoes and apples), more toxicological studies are warranted, both of the harvested commodities during storage and transport and of their derived products. Surveillance of fruit juices and other fruit-derived products for the presence of *Alternaria* toxins is needed, in order to determine the level of human exposure resulting from these products, and to evaluate the effort needed for its suppression.

In order to take the overall hazards of infected fruits and vegetables into account, it is also necessary to undertake an overall examination of *Alternaria* phytotoxins, because of a possible correlation between plant and animal toxicity. There is a need for more toxicological studies of purified *Alternaria* toxins, including studies of possible carcinogenic activity. It is important to estimate human exposure to *Alternaria* mycotoxins by examining either single toxins or combinations of toxins similar to those that occur naturally in some fruits during prolonged storage.

Studies of the fungal population genetics and relationships amongst *A. alternata* isolates and evaluation of the part played by the *A. tenuissima* species-group as mycotoxin producers in fruits and vegetables are among other matters that merit attention.

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# Molecular Diversity of *Aspergillus* and *Penicillium* Species on Fruits and Vegetables

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## ABSTRACT

*Aspergilli* and *penicillia* are frequently encountered on fruits and vegetables. The most notorious plant pathogens are *Penicillium expansum*, *Aspergillus niger* and *A. flavus*, which may cause various plant diseases. Although only a limited number of *Aspergillus* and *Penicillium* species can invade living plant tissues, they are frequently encountered as post-harvest contaminants of agricultural products. *Aspergilli* and *penicillia* can contaminate fruits and vegetables at different stages including harvest, processing and handling. Post-harvest contamination can lead to changes in the quality and nutritional value of fruits and vegetables. The most important aspect of food spoilage caused by these organisms is the formation of mycotoxins, which may have harmful effects on human and animal health. Mycotoxin contamination of fruits including grapes, figs, treenuts and peanuts is the economically most important. In this review, we attempt to give an overview on the genetic diversity and mycotoxin production abilities of *Penicillium* and *Aspergillus* species on fruits and vegetables.

## 9.1. INTRODUCTION

*Aspergillus* and *Penicillium* are among the most economically important fungal genera. *Aspergillus* isolates are used for the production of soy sauce, several



organic acids and enzymes, and biologically active metabolites such as lovastatin (Campbell-Platt and Cook, 1989; Bennett and Klich, 1992; Pariza and Johnson, 2001; Manzoni and Rollini, 2002), while penicillia are used in the pharmaceutical industry to produce penicillin, and in the food industry for the production of various cheeses and salami (Frisvad and Samson, 2004). Although not considered to be major causes of plant disease, both *Aspergillus* and *Penicillium* species may be responsible for several disorders in various plants (Tables 9.1 and 9.2). The most notorious plant pathogens are *P. expansum*, *A. niger* and *A. flavus*, which may cause various plant diseases. In contrast with specialized plant pathogens such as powdery mildews, rusts or most *Fusarium* species, these species are opportunistic pathogens without host specialization as proved in *A. flavus* (St. Leger et al., 2000).

While only a limited number of *Aspergillus* and *Penicillium* species can invade living plant tissues, they are frequently encountered as post-harvest contaminants of agricultural products (Raper and Fennell, 1965; Kozakiewicz, 1989). Aspergilli and penicillia can contaminate fruits and vegetables at different stages including harvest, processing and handling. Changes due to spoilage by these species can be of a sensory nature, e.g. pigmented growth, discoloration, rotting, development of off-odors and off-flavors. The most important aspect of food spoilage caused by these organisms is, however, the formation of mycotoxins, which may have harmful effects on human and animal health. Several mycotoxins have been identified which may

TABLE 9.1 *Aspergillus* species involved in plant pathogenesis (compiled from Raper and Fennell, 1965; Kozakiewicz, 1989; Michailides et al., 2002, [www.apsnet.org](http://www.apsnet.org)).

Plant	Disease	Species involved
Almond	Kernel decay	<i>A. niger</i> , <i>A. flavus</i> , <i>A. parasiticus</i>
	Chlorosis	<i>A. niger</i>
Apples	Fruit rot	<i>A. sclerotiorum</i> , <i>A. terreus</i>
Apricot, peach	Ripe fruit rot	<i>A. niger</i>
Carrot	Sooty rot	<i>A. niger</i>
Citrus ( <i>Citrus</i> spp.)	Albinism	<i>A. flavus</i>
	Black mold rot	<i>A. niger</i>
	Minor ear rots	<i>A. niger</i> , <i>Aspergillus</i> sp.
	Fruit and root rot	<i>A. flavus</i>
Date Palm	Fruit rots	<i>Aspergillus</i> sp.
Fig	Fig smut	<i>A. niger</i>
Grape	Vine canker	<i>A. niger</i>
	Bunch rot (sour rot)	<i>A. niger</i>
	Berry rots	<i>A. aculeatus</i> , <i>Aspergillus</i> sp.
Mango	Black mold rot	<i>A. niger</i>
Onion, garlic	Black rot	<i>A. niger</i> , <i>A. alliaceus</i>
Peanut	Crown rot	<i>A. niger</i>
Pineapple	<i>Aspergillus</i> rot	<i>A. flavus</i>
Pistachio	<i>Aspergillus</i> fruit rot	<i>A. niger</i>
Strawberry	Fruit rots	<i>A. niger</i>

TABLE 9.2 *Penicillium* species causing plant decay.

Plants		Species (disease caused)
Fruits	Citrus fruits	<i>P. italicum</i> (=blue mold rot)(Snowdon, 1990); <i>P. digitatum</i> (=green mold rot) (Snowdon, 1990); <i>P. ulaiense</i> (Whisker mold) (Samson and Frisvad, 2004)
	Pine apple	<i>P. funiculosum</i> (=interfruitlet corking, leathery pocket) (Snowdon, 1990); <i>Talaromyces macrosporus</i> (Frisvad et al., 1990)
	Pome fruits (apples and pears)	<i>P. expansum</i> (=blue mold rot); <i>P. funiculosum</i> (=core rot and moldy core) (Snowdon, 1990); <i>P. crustosum</i> ; <i>P. solitum</i> (Samson and Frisvad, 2004)
	Stone fruits (peaches, nectarines, apricots, plums and cherries)	<i>P. expansum</i> (=blue mold rot) (Snowdon, 1990); <i>P. griseofulvum</i> (Samson and Frisvad, 2004)
	Soft fruits (strawberries, raspberries)	<i>P. expansum</i> (=blue mold rot) (Snowdon, 1990)
	Grapes	<i>P. canescens</i> , <i>P. citrinum</i> , <i>P. cyclopium</i> (Snowdon, 1990), <i>P. expansum</i> (Frisvad et al., 2007); <i>P. carneum</i> (El Khoury et al., 2006)
	Kiwifruits	<i>Penicillium</i> sp. (=blue mold rot) (Snowdon, 1990)
	Misc. tropical and subtropical fruits	<i>P. expansum</i> (=blue mold rot) (Snowdon, 1990)
Beans and peas	Black beans, cowpeas	<i>P. citrinum</i> (Frisvad et al., 2007); <i>P. aethiopicum</i> ; <i>P. viridicatum</i> (Samson and Frisvad, 2004)
Nuts	Almonds, hazelnuts, pistachio, walnuts	<i>P. crustosum</i> ; <i>P. discolor</i> (Frisvad et al., 2007); <i>P. expansum</i> ; <i>P. solitum</i> (Samson and Frisvad, 2004)
Oil crop Vegetables	Peanut	<i>P. polonicum</i> (Samson and Frisvad, 2004)
	Olives	<i>P. citrinum</i> ; <i>P. expansum</i> (Frisvad et al., 2007)
	Ginger	<i>P. brevicompactum</i> (Frisvad et al., 2007)
	Onions	<i>P. glabrum</i> (Frisvad et al., 2007); <i>P. albocoremium</i> ; <i>P. allii</i> ; <i>P. radicicola</i> ; <i>P. tulipae</i> (Samson and Frisvad, 2004)
	Garlic	<i>P. allii</i> (=blue mold rot) (Frisvad et al., 2007)
	Tomatoes	<i>P. expansum</i> ; <i>P. olsonii</i> ; <i>P. tularense</i> (Frisvad et al., 2007)
	Yams	<i>P. sclerotigenum</i> (Frisvad et al., 2007)
	Brussels sprouts	<i>P. bialowiezense</i> (Samson and Frisvad, 2004)
	Carrots	<i>P. radicicola</i> (Samson and Frisvad, 2004)
	Potatoes	<i>P. radicicola</i> (Samson and Frisvad, 2004)

TABLE 9.3 Some economically important mycotoxins produced by *Aspergillus* species in various agricultural products (Larsen et al., 2001, Bayman et al., 2002b, Varga et al., 2003a,b; Frisvad et al., 2004, Paterson et al., 2003).

Mycotoxins	Agricultural product	Species
Aflatoxins	Peanut, corn, cotton	<i>A. flavus</i> , <i>A. parasiticus</i> , <i>A. nomius</i>
	Dried fruits (dates)	
Ochratoxins	Cereals	<i>P. verrucosum</i>
	Meat, cheese	<i>P. nordicum</i>
	Grape, wine	<i>A. niger</i> , <i>A. carbonarius</i>
	Coffee, spices	<i>A. ochraceus</i> , <i>A. steynii</i> , <i>A. westerdijkiae</i> ,
		<i>A. niger</i> , <i>A. carbonarius</i>
Patulin	Figs	<i>A. alliaceus</i> , <i>A. niger</i>
	Cereals, malt	<i>P. expansum</i> , <i>A. clavatus</i>
	Apple, pear	<i>P. expansum</i> , <i>P. roquefortii</i> , <i>P. brevicompactum</i>

contaminate foods including fruits and vegetables, the economically most important of which are aflatoxins, ochratoxins and patulin (Table 9.3).

In this review, we will give an overview on the occurrence and genetic diversity of *Aspergillus* and *Penicillium* species on fruits and vegetables.

## 9.2. OCCURRENCE AND GENETIC VARIABILITY OF ASPERGILLI AND PENICILLIA ON FRUITS

### 9.2.1. GRAPES

*Aspergillus* species have been found to be responsible for only a limited number of preharvest plant diseases (Table 9.1). Among these, contamination of grapes is one of the most important as aspergilli responsible for this disorder may produce mycotoxins including ochratoxin A (OTA), which can contaminate grapes and turn up in grape juices and wine (Varga and Kozakiewicz, 2006). Recent studies have focused on identifying the source of OTA in wines in several countries. Data suggests that OTA contamination in Mediterranean countries as well as in subtropical parts of Brazil, Argentina and Australia is caused by black aspergilli (Table 9.4); Varga and Kozakiewicz, 2006). Black aspergilli are the causal agents of several plant diseases, and considered as opportunistic pathogens of grape and may cause bunch rot (sour rot) or berry rots, and raisin mold rot (Varga et al., 2004). This disease is particularly severe in warmer grape-growing areas including southern parts of Spain, Portugal, France, Italy, Greece, Morocco and Egypt (Logrieco et al., 2003). A recent study indicated that black aspergilli are also responsible for vine canker of grapes (Michailides et al., 2002). Cozzi et al. (2006) have recently suggested that larvae of the grape berry moth (*Lobesia botrana*) serve as vectors for OTA-producing black aspergilli, leading to OTA accumulation in berries.

TABLE 9.4 Fungi responsible for ochratoxin contamination of grapes and wine in different countries.

Country	Species responsible	Reference
Argentina	<i>A. niger</i> aggregate	Chulze et al., 2006
Brazil	<i>A. niger</i> , <i>A.ochraceus</i> , <i>A. carbonarius</i>	Chulze et al., 2006
Australia	<i>A. niger</i> , <i>A. carbonarius</i> , ( <i>A. aculeatus</i> <sup>a</sup> )	Leong et al., 2006
France	<i>A. carbonarius</i> , <i>A. niger</i> aggregate	Bejaoui et al., 2006
Lebanon	<i>A. carbonarius</i>	El Khoudy et al., 2006
Greece	<i>A. carbonarius</i> , <i>A. niger</i> aggregate	Tjamos et al., 2006
Hungary	<i>A. niger</i> aggregate	Varga and Kozakiewicz, 2006
Israel	<i>A. niger</i> aggregate, <i>A. carbonarius</i>	Guzev et al., 2006
Italy	<i>A. niger</i> aggregate, <i>A. carbonarius</i>	Battilani et al., 2006
Portugal	<i>A. carbonarius</i> , <i>A. niger</i> aggregate, <i>A. ochraceus</i> <sup>b</sup>	Abrunhosa et al., 2001; Serra et al., 2003, 2006b
Spain	<i>A. niger</i> aggregate, <i>A. carbonarius</i> , <i>A. ochraceus</i>	Belli et al., 2006
Tunisia	<i>A. niger</i> aggregate, <i>A. carbonarius</i>	Lasram et al., 2007
Czech Republic	—	Malir et al., 2006

<sup>a</sup> Ochratoxin A production by this species has not been confirmed  
<sup>b</sup> Many strains previously identified as *A. ochraceus* have later been shown to be *A. westerdijkiae* (Frisvad et al., 2004)

Among the black aspergilli, *A. carbonarius* is the most important species as OTA-producing isolates are observed most frequently (Téren et al., 1996; Abarca et al., 2003; Battilani et al., 2003). Apart from *A. carbonarius*, *A. niger* has also been found to produce OTA on grapes (Battilani et al., 2003, 2006c). Recently, Medina et al. (2005) and Perrone et al. (2006) claimed OTA production in *A. tubingensis* isolates originating from grapes. There are also some few reports on OTA production by *A. aculeatus* (Battilani et al., 2003), but this has not been confirmed in other studies (Guzev et al., 2006). Besides OTA-producing species, other black aspergilli including *A. ibericus*, *A. brasiliensis*, *A. japonicus* and *A. aculeatus* have also been identified on grapes (Serra et al., 2006a,b). In a recent 3-year study, more than 2114 black *Aspergillus* isolates that came from grapes were analyzed for OTA production (Guzev et al., 2006). While about 3 percent of isolates belonging to the *A. niger* species aggregate produced OTA, 35 percent of *A. carbonarius* isolates were found to be OTA producers. None of the uniseriate species produced OTA in detectable quantities. Battilani et al. (2006a,b) observed a West–East and North–South gradient in the incidence of *Aspergillus* section *Nigri* and *A. carbonarius* isolates in Southern Europe and Israel in a 3-year period (2001–2003). The highest incidences have been observed in Israel, Greece and Southern France, associated with the highest incidence of *A. carbonarius*. Southern Spain and Southern Italy also had relevant incidence of black aspergilli. The authors also observed that meteorological conditions can contribute to explain spatial distribution variation of black aspergilli within the Mediterranean basin.

Due to the ability of *A. carbonarius* and *A. niger* to produce OTA at a wide range of temperatures, OTA can be continuously produced in the field. This fact has to be taken into account in commodities such as grapes, raisins and wine, where *A. carbonarius* and members of the *A. niger* aggregate are considered to be the main sources of the OTA contamination. Ochratoxin A contamination of dried vine fruits was also found to be due to the action of black aspergilli in Europe including Spain (Abarca et al., 2003), the Czech Republic (Ostry et al., 2002), Hungary (Varga et al., 2006) and in other parts of the world including Argentina (Magnoli et al., 2004; Romero et al., 2005) and Australia (Leong et al., 2004).

In countries with colder temperate climates such as Germany, Northern Hungary, the Czech Republic or Northern parts of Portugal, France and Italy, black aspergilli have not been isolated from grape berries in spite of the presence of OTA in wines (Zimmerli and Dick, 1996; Abrunhosa et al., 2001; Torelli et al., 2003; Ostry et al., 2004; Varga et al., 2005; Varga and Kozakiewicz, 2006). In colder climates, *P. verrucosum* was found to be responsible for OTA contamination of several agricultural products including cereals (Lund and Frisvad, 2003), and in meat products *P. nordicum* is the dominant OTA producer (Larsen et al., 2001; Frisvad and Samson, 2004). No other well-documented OTA producers are known. Penicillia are able to contaminate grapes as found by Tournas and Katsoudas (2005), and Serra et al. (2006b). Battilani et al. (2001), Torelli et al. (2003) and Rousseau (2004) claimed to have found OTA-producing *Penicillium* species from grapes in Northern Italy and France, suggesting that *Penicillium* species could be responsible for OTA contamination of grapes in these regions. Indeed, Torelli et al. (2006) claimed OTA-producing *Penicillium* isolates from grapes used for the production of 'passito' wines in Italy, as they found no *Aspergillus* species in these grapes. However, species identification or ochratoxin production was not convincingly confirmed.

The two well-known producers of OTA, *P. verrucosum* and *P. nordicum*, have never been found in grapes, but other *Penicillium* species were detected such as *P. expansum* producing citrinin and patulin in pure culture. Only one strain out of 51 of *P. expansum* could produce citrinin in grape juice, whereas 33 strains out of 51 could produce patulin (Abrunhosa et al., 2001).

Some penicillia are able to produce geosmin, a volatile compound giving fungal or earthy odor to wine (Larsen and Frisvad, 1995). Two species able to produce this compound, *P. expansum* and *P. carneum*, have been identified on grapes recently (La Guerche et al., 2005). In a recent paper, El Khoury et al. (2006) observed aflatoxin producing *A. flavus* isolates on Lebanese grapes, indicating that grape juices and wine could also be contaminated by aflatoxins.

### 9.2.2. POMACEOUS AND STONE FRUITS

Pomaceous and stone fruits can be degraded by a number of pathogenic species including *Monilia laxa*, *M. fructigena* and *Rhizopus stolonifer*. *Alternaria alternata*,

*Alt. tenuissima* species-group, *Fusarium lateritium*, *P. expansum*, *P. crustosum* and *P. solitum* were reported as toxigenic organisms and are also able to produce rot in apples (Raper and Thom, 1949; Samson et al., 1976; Frisvad, 1981; Reuveni et al., 2002; Serdani et al., 2002; Andersen and Thrane, 2006). The *Alternaria arborescens* species-group and *P. expansum* are commonly isolated from cherry (Roberts et al., 2000; Andersen and Thrane, 2006). In Ya Li pear fruit *Alt. yaliinficiens* was reported as a severe fruit spoiler (Roberts, 2005).

*Penicillium expansum* is known for its production of the mycotoxins patulin and citrinin and these have been found in moldy fruits (Harwig et al., 1973; Ciegler et al., 1977; McKinley and Carlton, 1991; Vinas et al., 1993). Naturally occurring mycotoxins, such as patulin, chaetoglobosins, communesin B and roquefortine C, have been detected in windfall apples and in an apple still on the tree (Paterson et al., 2003; Andersen et al., 2004).

*Aspergillus* species may also cause a rot in apples, especially *A. sclerotiorum* (Huber, 1933) and *A. terreus* (Tandon and Bhatnagar, 1958).

### 9.2.3. CITRUS

Citrus trees (lemons, oranges, mandarins, tangerine, cumquats, tangelo, limes, pomelos, grapefruits) are susceptible to many different plant pathogenic *Alternaria* species attacking leaves (Simmons, 1999; Peever et al., 2002) depending on citrus cultivar.

In storage three spoilage penicillia are of paramount importance: *P. digitatum*, *P. italicum* and *P. ulaiense* (Birkinshaw et al., 1931; Raper and Thom, 1949; Westerdijk, 1949; Holmes et al., 1994). According to Holmes et al. (1994), *P. ulaiense* only appears when the other two pathogens are inhibited by fungicides and is much more related to *P. italicum* than to *P. digitatum* (Samson et al., 2004).

Germination of *P. digitatum* conidia on citrus, which are non-climacteric fruits, is stimulated by certain combinations of the volatiles surrounding wounded fruit, notably limonene ( $\alpha$ -pinene, sabinene,  $\beta$ -myrcene, acetaldehyde, ethanol, ethylene and CO<sub>2</sub>). Ethylene did not stimulate the germination of *P. digitatum* conidia in the non-climacteric fruit (Eckert and Ratnayake, 1994), whereas ethylene invited fungal attack in the climacteric tomatoes, avocados and bananas (Flaishman and Kolattukudy, 1994). Other components in oranges, such as simple sugars and organic acids, also stimulate conidium germination in *P. digitatum* (Pelser and Eckert, 1977). Furthermore, a few fruits spoiled by fungi can cause reduced shelf life of the sound fruits due to accelerated ripening or senescence triggered by releasing the gas ethylene (Rippon, 1980). This shows that the associated mycobiota can tolerate and sometimes can even be stimulated by the acids and other protecting volatile and non-volatile phytoalexins of citrus fruits in combination with the ability to produce pectinases and other citrus skin degrading enzymes. The fungal activities result in serious weight loss, shrinkage and softening of the citrus fruits (Ben-Yehoshua et al., 1987).

Toxic metabolites from *P. italicum* and *P. digitatum* have never been found in citrus fruits, yet these fungi produce compounds that are toxic to bacteria, plants, brine shrimps and chick embryos (Faid and Tantaoui-Elaraki, 1989). One *P. italicum* isolate that was toxic in laboratory animals (Kriek and Wehner, 1981) was found to produce the mycotoxin 5,6-dihydro-4-methoxy-2H-pyran-2-one (Gorst-Allman et al., 1982) related to verrucolone (arabenoic acid) (Isaac et al., 1991; Larsen et al., 1998). Several other *P. italicum* secondary metabolites have been identified, but not tested for toxicity (Arai et al., 1989). *P. digitatum* has been found to produce tryptoquialanins and tryptoquialanons (Ariza et al., 2002; Frisvad and Samson, 2004).

#### 9.2.4. FIGS

Another fruit frequently contaminated by aspergilli is fig. Fig smut is usually associated with black aspergilli colonizing the ripen fruits, but other species including *A. flavus*, *A. alliaceus* and *A. ochraceus* have also been detected on decayed fruits (Buchanan et al., 1975; Doster et al., 1996). Immature fig fruits did not support colonization and aflatoxin production by *A. flavus*, and became susceptible when ripe (Buchanan et al., 1975). The black-spored *Aspergillus* isolates that caused the disease fig smut were classified as *A. niger*, *A. tubingensis*, *A. japonicus*, and *A. carbonarius* (Doster et al., 1996; Bayman et al., 2002a). The presence of aspergilli on figs can lead to accumulation of mycotoxins including OTA and aflatoxins (Doster et al., 1996). Aflatoxin-producing *A. flavus* isolates are frequently encountered in figs (Buchanan et al., 1975; Boudra et al., 1994), while ochratoxin accumulation was caused mainly by *A. alliaceus* isolates in fig orchards in California (Bayman et al., 2002b). Figs infected with *A. alliaceus* contained OTA, while figs infected with *A. ochraceus*, *A. melleus* or *Penicillium* species had little or none, suggesting that *A. alliaceus* may be responsible for the ochratoxin contamination occasionally observed in figs in California.

#### 9.2.5. PINEAPPLE

Several species, including *P. funiculosum* and *Talaromyces macrosporus*, can grow on pineapples and cause pineapple black spot, core rot and post-harvest decay. The only known mycotoxin from *T. macrosporus* is duclauxin (Frisvad et al., 1990), but it has never been found in pineapple itself.

#### 9.2.6. TREE NUTS

Tree nuts, including almond, hazelnut, walnut and pistachio nut, are frequently contaminated by *Aspergillus* species including potentially mycotoxigenic black aspergilli, *A. flavus*, *A. parasiticus* and *A. ochraceus*. In the case

of pistachio nuts, kernels infested by the insect navel orange worm (*Amye-lois transitella*) had substantially more infections and produced 84 percent of all the aflatoxin detected (Doster and Michailides, 1994). The same authors found a correlation between hull splitting, shell discoloration and infection rate in dates (Doster and Michailides, 1995, 1999). In another study, *A. niger* was isolated most frequently from almonds, pistachios and walnuts (Bayman et al., 2002a). Ochratoxin producing *A. alliaceus* isolates were suggested to be responsible for the occasional OTA contamination of tree nuts (Bayman et al., 2002b). Ochratoxin producing *A. lanosus*, *A. sclerotiorum* and *A. melleus* isolates have also been found (Bayman and Baker, 2006). Other potentially mycotoxigenic species identified included *A. flavus*, *A. ochraceus*, *A. alliaceus*, *A. melleus* and *A. fumigatus*. In a European study, *A. flavus* and *A. parasiticus* have been identified in almond, pistachio and hazelnut samples (Jimenez and Mateo, 2001). Other species detected include black aspergilli, *A. ochraceus*, *A. fumigatus*, *A. candidus* and *A. versicolor*.

Control of insect pests in tree nut orchards is a major strategy to lower aflatoxin contamination, since insect feeding damage can lead to fungal infection and subsequent mycotoxin contamination. Through breeding and genetic engineering, new varieties of almonds and walnuts have been developed which are resistant to insect attack (Campbell et al., 2003).

## 9.2.7. OTHER FRUITS

Other diseases of fruits including mango rot (Vala et al., 1989), rot of date fruit (Brown, 1920), citrus fruit rot ([www.apsnet.org](http://www.apsnet.org)), peach rot (Michailides and Thomidis, 2007) and infection of black plum (Okigbo, 2001) can also be caused by aspergilli, mainly by *A. niger* and *A. flavus*. These are usually post-harvest diseases that occur during storage. Post-harvest decays of pears, apples, peaches, grapes, strawberries, melons and tomatoes are also frequently caused by these species (Barkai-Golan, 1980).

## 9.3. OCCURRENCE AND GENETIC VARIABILITY OF ASPERGILLI AND PENICILLIA ON VEGETABLES

### 9.3.1. TOMATO

Tomato plants are susceptible to different plant pathogenic *Alternaria* species attacking leaves (Simmons, 2000). Undamaged tomatoes are quite resistant to fungal spoilage, but *P. tularense* and *P. expansum* have been reported on healthy tomatoes (Andersen and Frisvad, 2004). *P. olsonii* has often been found on damaged tomatoes and may grow directly on commercial tomatoes, but no mycotoxins have been reported from this species.



### 9.3.2. ONION AND GARLIC

Apart from *Botrytis aclada*, few species are able to spoil garlic and onion (Abdel-Sater and Eraky, 2002). *P. allii* is a widespread spoiler of garlic, causing the blue mold rot (Frisvad and Filtenborg, 1989; Vincent and Pitt, 1989). Symptoms of the disease are yellow spots on cloves, with blue conidial masses developing as the disease progresses. The closely related *P. albocoremium*, *P. radicola* and *P. tulipae* are more common on onions (Overy and Frisvad, 2003; Overy et al., 2005a,b). *Petromyces alliaceus*, *A. niger* and *P. glabrum* are cited as producers of rots in onions (Raper and Thom, 1949; Raper and Fennell, 1965; Hayden and Maude, 1994; Hayden et al., 1994). *P. glabrum* appears to grow only in the outer layers of onions.

Black mold rot is caused by black aspergilli, and affects mainly onions. The primary symptom of this disease is a black discoloration of tissue. Infected bulbs may show blackening at the neck, black streaks or spots on or beneath the outer scales, and black discoloration in injured areas. Seedlings can become infected either from fungus within the seed or when clean seed is planted in contaminated soil. In addition to occurring within the seed, this fungus can also be present throughout the plant (in roots, basal plate and leaves). This infection can be symptomless until the plants become mature, indicating that the fungus is capable of becoming endophytic (alive but not producing disease symptoms) in seedlings and young onion plants (Lorbeer, 1998). High temperatures and moisture levels at maturity result in the production of the black mold symptoms (Lorbeer, 1998). *A. niger* can mainly be found in the seed coat (Koycu and Ozer, 1997). Contamination from bulb to bulb progresses rapidly during storage (Raper and Fennell, 1965; Mundt, 1978; Ryall and Lipton, 1979; Filtenborg et al., 1996).

Hungarian onions have been found to be infected by *A. niger* belonging to mitochondrial DNA groups 1b and 1c (Varga et al., 1993, 1994). Some of these isolates have also been found to produce OTA (unpublished data). Another mycotoxin produced by black aspergilli in onions is malformin (Curtis et al., 1974). Malformin was found in the outer scales of onion, not in central portions of the bulb. Other species frequently encountered on onions are *A. flavus*, *A. alliaceus* and *A. fumigatus* (Walker and Lindegren, 1924; Ozer and Koycu, 2004).

Different onion cultivars were found to differ in their susceptibilities to black mold (Sinclair and Letham, 1996; Ko et al., 2002). These observations indicate that breeding could be an effective tool to protect onions and garlic from infection.

### 9.3.3. GINGER

Ginger is resistant to most fungal attacks. However *P. brevicompactum* has been reported to grow and produce mycophenolic acid in this root (Overy and Frisvad, 2005).

### 9.3.4. PEANUTS

Contamination of peanut by *Aspergillus* species leading to the death of several thousand turkeys led to the discovery of aflatoxins in the early sixties (Lancaster et al., 1961). Since then, huge amounts of research have been carried out on fungal colonization and aflatoxin contamination of peanuts. Aflatoxin contamination of peanuts causes huge economical losses in several countries including Brazil, Argentina and Australia (Rodriguez-Amaya and Sabino, 2002; Scussel, 2004; Pitt and Hocking, 2006). Peanut plants can be invaded by *A. flavus* and *A. parasiticus* from soil or seed as early as the time of emergence from the soil (Pitt et al., 1991). The fungi can spread throughout the plants, though prevalence is higher in parts nearer the soil.

Aflatoxigenic *A. flavus* and *A. parasiticus* isolates frequently occur in Spanish peanuts (Jimenez and Mateo, 2001). Other potentially toxigenic species identified include *A. ochraceus*, black aspergilli, *Eurotium chevalieri*, *E. repens*, *A. fumigatus* and *A. terreus* (Jimenez and Mateo, 2001). Recent studies of the genetic diversity of aspergilli isolated from peanuts and peanut fields in Argentina indicate *A. flavus* populations exhibit high levels of variability, while *A. parasiticus* populations are less diverse (Pildain et al., 2004; Barros et al., 2005, 2006a,b). In another study, *A. flavus* was found to be the dominant species in peanut fields sampled across the United States (Horn and Dörner, 1998). Besides *A. parasiticus*, *A. tamarii* and *A. caelatus* also occurred at lower densities. Peanut fields had significantly higher densities and incidences of *A. parasiticus* than fields planted with other crops. High levels of diversity of vegetative compatibility groups in *A. flavus* were found on the peanut fields (Horn and Greene, 1995). The authors suggested that this diversity might be caused by aerial spore dispersal from infected corn and cotton fields. McAlpin et al. (2002) developed a DNA fingerprinting method for the identification of *Aspergillus* Section *Flavi* isolates in peanut fields. Using this method, high degrees of variability could be observed in *A. caelatus* isolates collected in peanut fields (McAlpin et al., 2005).

Resistance to fungal colonization and aflatoxin contamination was found to be associated with seed coat integrity in some peanut genotypes (Asis et al., 2005). Both classical and molecular breeding approaches are in progress to develop peanut lines resistant to *A. flavus* infection (Wynne et al., 1991; Anderson et al., 1996; Ozias-Akins et al., 2002).

### 9.3.5. OTHER VEGETABLES

Crucifers including cabbages, cauliflower, radish, cress and turnip are also prone to *A. niger* contamination (Abdel-Mallek, 1995; Lugauskas et al., 2005). Many vegetables are often infected and spoiled by bacteria, resulting in a low fungal count (Tournas, 2005). *Aspergillus* spp., e.g. *A. flavus*, *A. niger*, *A. fumigatus* or penicillia (*P. oxalicum*), often occur in dried vegetables. *Penicillium discolor* have also been identified on various vegetables (Frisvad et al., 1997).

## 9.4. CONCLUSIONS

Species of *Aspergillus* and *Penicillium* are generally not known as plant pathogens causing field diseases. However, because of the production of numerous conidia, they can easily infect fruits and vegetables and develop as post-harvest contaminants. Each species in *Aspergillus* and *Penicillium* genera has its specific habitat and many data on their association with fruits and vegetables are available. Research has also shown that each taxon is characterized by its specific extrolite pattern and the production of mycotoxin(s). Therefore it is essential that a correct identification of *Penicillium* and *Aspergillus* species should be carried out so information about a possible health hazard can be considered.

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# Detection of *Penicillium*, *Aspergillus* and *Alternaria* Species in Fruits and Vegetables

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## ABSTRACT

Molds are widely distributed in nature occurring on a large variety of agricultural commodities, including vegetables, fruits, cereals, nuts, and spices. Mold contamination results in quality deterioration of foods and feeds. Moreover, mycotoxins, the secondary metabolites produced by molds, can adversely affect human and animal health. Therefore accurate methods for detection and quantitation of molds and mycotoxins are of economic and public health significance. In addition, valid assays for detecting mycotoxigenic molds and mycotoxins in foods are required for implementing control and regulatory strategies. This has led to the development of several accurate and sensitive analytical techniques for detection of molds and their toxins in foods. This chapter briefly summarizes the various mold species and mycotoxins of significance in fruits and vegetables, and discusses the conventional and emerging detection methods for molds. The major challenge for the emerging technologies is to demonstrate advantages over the conventional and established techniques for application at various stages of crop and food production.

## 10.1. INTRODUCTION

Molds are ubiquitous in nature. They are natural inhabitants of soil and are contaminants of air, water, foods, and feeds. Mold contamination does not only cause quality deterioration of foods and feeds, but also can

adversely affect human and animal health due to the production of toxic metabolites called mycotoxins (Frisvad and Filtenborg, 1983; El-Banna et al., 1987; Chelkowski, 1998). The production of mycotoxins occurs in the field, and during distribution, processing, and storage of foods depending on environmental factors and storage conditions. The various environmental factors affecting mold growth and mycotoxin production include temperature, pH, moisture content, oxygen levels, carbohydrates, trace elements, the mold strains, and microbial competition. Although there is no direct relationship between the presence of molds on food and crops and the presence of mycotoxins, the absence of mold fragments or mold spores does not guarantee that foods and crops are free of mycotoxins. This is because mycotoxins are heat resistant and can survive for long periods even after the molds have been destroyed or inactivated. Therefore timely detection of molds on foods and crops is critical to avoid potential health hazards.

Historically, molds have been detected either visually or indirectly by the effects they cause on foodstuffs as they grow. Traditional mold identification procedures are based primarily on the recognition of the characteristics of the colony and micromorphology, dilution plating, measurements of CO<sub>2</sub> levels and volatile compounds, ATP assays, and an evaluation of the presence of fungal cell constituents such as ergosterol and chitin (Cousin, 1996). More recently, the tools of molecular biology have led to the development of specific and sensitive methods for the rapid identification of toxigenic molds on foods. Nucleic acid-based detection methods such as RAPD (Random Amplified Polymorphic DNA) (Niessen et al., 2005), Amplified Fragment Length Polymorphism (AFLP) (Niessen et al., 2005) Restricted Fragment Length Polymorphism (RFLP) (Niessen et al., 2005), real-time quantitative PCR (Niessen et al., 2005), multiplex PCR (Yang et al., 2004), and PCR-ELISA (Grimm and Geisen, 1998) have been developed for the detection of major mycotoxigenic species associated with fruits and vegetables. In addition, immunological methods such as Enzyme Linked Immuno Sorbent Assay (ELISA) have been used for detecting molds (Clarke et al., 1986; Banks et al., 1994). The ELISA-based methods involve detection of mycelial washings or exoantigens (Lu et al., 1995a,b) or heat-stable polysaccharides (Notermans and Heuvelman, 1985; Van der Horst et al., 1992). Among ELISA, a few non-specific assays have also been developed to detect molds on foods by using polyclonal antibodies to a mixture of common food-borne species (Robertson et al., 1986; Yong and Cousin, 1995). This chapter will focus on the various mold species and mycotoxins of significance in fruits and vegetables, and their detection methods. Table 10.1 summarizes the major mycotoxin-producing molds on fruits and vegetables, which include *Aspergillus*, *Penicillium*, and *Alternaria* (Gourama and Bullerman, 1995; Pitt and Hocking, 1997).

Organism	Mycotoxin	Affected fruit/vegetable	References
<i>A. parasiticus</i> <i>A. flavus</i> <i>A. niger</i> <i>A. oryzae</i>	Aflatoxins B1, B2, G1, G2, M1	Oranges, grapefruits, figs, tomatoes, cereal grains, nuts, beans, dry fruits, corn, peanuts, cotton seed	Ragab et al., 1999; Varma and Verma, 1987; Alderman and Marth, 1974; Bankole, 1993; Buchanan et al., 1975; Selim et al., 1996
<i>Alternaria alternata</i> , <i>A. tenuissima</i> , <i>A. solani</i>	Alternariol, Alternariol methyl ether, Tenuazonic acid	Tomatoes, apples, strawberries, blueberries, grapes, oranges, lemons, peppers, leafy crucifers, wheat, sorghum	Palmisano et al., 1989; Logrieco et al., 1990; Tournas and Stack, 2001; Stinson et al., 1980; Stinson et al., 1981; Scott, 2001
<i>A. ochraceus</i> , <i>P. citrinum</i>	Citrinin	Coconuts, apples, rice, barley, maize	Frank, 1992; Nishijima, 1984
<i>P. citreonigrum</i> <i>P. ochrosalmoneum</i> <i>P. citreoviride</i> <i>P. charlesii</i>	Citreoviridin	Maize, rice, corn	Abramson, 1977; Ueno and Ueno, 1972
<i>P. cyclopium</i> <i>A. flavus</i>	$\alpha$ -Cyclopiazonic acid	Maize, peanuts, millets, tomatoes, corn flakes	Urano et al., 1992; Rao and Hussain, 1985; da Motta and Soares, 2001; Aresta et al., 2003
<i>A. ochraceus</i> <i>P. verrucosum</i> <i>A. fumigatus</i> <i>A. versicolor</i> <i>A. alliaceus</i>	Ochratoxin A	Onions, garlics, cocoa, coffee, dried fruits, cereals	Abarca et al., 2001; Hesselstine et al., 1972; Sage et al., 2002
<i>P. expansum</i> <i>P. patulum</i> <i>P. claviforme</i> <i>Aspergillus clavatus</i> <i>Alternaria alternata</i>	Patulin	Apples, apple juice, grape juice, peaches, strawberries, greengage tomatoes, bananas, cherries, pears, olives, apricots, cereals	Lovett et al., 1974; Paster et al., 1995; Askar and Siliha, 1992; Arici, 2000; Abrunhosa et al., 2001
<i>Penicillium puberulum</i> <i>Penicillium</i> spp. <i>Aspergillus ochraceus</i>	Penicillic acid	Maize, beans, peanuts, tree nuts	Jelinek et al., 1989
<i>Aspergillus versicolor</i> , <i>A. flavus</i> , <i>S. sydowi</i> , <i>A. nidulans</i>	Sterigmatocytin	Grains, coffee beans, maize, fruits, grape juice	Davis, 1981; Terao, 1983; Pande et al., 1990; Scudamore et al., 1996

Organism	Mycotoxin	Affected fruit/vegetable	References
<i>A. parasiticus</i> <i>A. flavus</i> <i>A. niger</i> <i>A. oryzae</i>	Aflatoxins B1, B2, G1, G2, M1	Oranges, grapefruits, figs, tomatoes, cereal grains, nuts, beans, dry fruits, corn, peanuts, cotton seed	Ragab et al., 1999; Varma and Verma, 1987; Alderman and Marth, 1974; Bankole, 1993; Buchanan et al., 1975; Selim et al., 1996
<i>Alternaria alternata</i> , <i>A. tenuissima</i> , <i>A. solani</i>	Alternariol, Alternariol methyl ether, Tenuazonic acid	Tomatoes, apples, strawberries, blueberries, grapes, oranges, lemons, peppers, leafy crucifers, wheat, sorghum	Palmisano et al., 1989; Logrieco et al., 1990; Tournas and Stack, 2001; Stinson et al., 1980; Stinson et al., 1981; Scott, 2001
<i>A. ochraceus</i> , <i>P. citrinum</i>	Citrinin	Coconuts, apples, rice, barley, maize	Frank, 1992; Nishijima, 1984
<i>P. citreonigrum</i> <i>P. ochrosalmoneum</i> <i>P. citreoviride</i> <i>P. charlesii</i>	Citreoviridin	Maize, rice, corn	Abramson, 1977; Ueno and Ueno, 1972
<i>P. cyclopium</i> <i>A. flavus</i>	$\alpha$ -Cyclopiazonic acid	Maize, peanuts, millets, tomatoes, corn flakes	Urano et al., 1992; Rao and Hussain, 1985; da Motta and Soares, 2001; Aresta et al., 2003
<i>A. ochraceus</i> <i>P. verrucosum</i> <i>A. fumigatus</i> <i>A. versicolor</i> <i>A. alliaceus</i>	Ochratoxin A	Onions, garlics, cocoa, coffee, dried fruits, cereals	Abarca et al., 2001; Hesselstine et al., 1972; Sage et al., 2002
<i>P. expansum</i> <i>P. patulum</i> <i>P. claviforme</i> <i>Aspergillus clavatus</i> <i>Alternaria alternata</i>	Patulin	Apples, apple juice, grape juice, peaches, strawberries, greengage tomatoes, bananas, cherries, pears, olives, apricots, cereals	Lovett et al., 1974; Paster et al., 1995; Askar and Siliha, 1992; Arici, 2000; Abrunhosa et al., 2001
<i>Penicillium puberulum</i> <i>Penicillium</i> spp. <i>Aspergillus ochraceus</i>	Penicillic acid	Maize, beans, peanuts, tree nuts	Jelinek et al., 1989
<i>Aspergillus versicolor</i> , <i>A. flavus</i> , <i>S. sydowi</i> , <i>A. nidulans</i>	Sterigmatocytin	Grains, coffee beans, maize, fruits, grape juice	Davis, 1981; Terao, 1983; Pande et al., 1990; Scudamore et al., 1996

### 10.1.1. *ASPERGILLUS*

The genus *Aspergillus* contains several species capable of producing mycotoxins. Almost 50 species of *Aspergillus* have been reported as capable of producing toxic metabolites (Cole and Cox, 1981). However, the *Aspergillus* mycotoxins of greatest significance on foods and feeds are aflatoxins, ochratoxin A, sterigmatocystin, and cyclopiazonic acid. In addition, patulin, citrinin, and penicillic acid are also produced by some species of *Aspergillus* (Holzapfel, 1968; Davis, 1981; Terao, 1983; Jorgensen, 1998; MacDonald et al., 1999; Murphy et al., 2006).

#### 10.1.1.1. Aflatoxins

The aflatoxins are a group of mycotoxins produced by certain *Aspergillus* species, especially *Aspergillus flavus*, *A. parasiticus*, and *A. nominus*. The aflatoxins are extremely potent mutagens, suspected human carcinogens, and can adversely affect animal health and agricultural productivity. These mycotoxins occur in several chemical forms, designated as aflatoxin B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub>, and M<sub>1</sub> (Murphy et al., 2006). The most potent and frequently occurring of these compounds is aflatoxin B<sub>1</sub> (Richard et al., 1993).

Aflatoxins often occur naturally on crops in the field prior to harvest. Post-harvest contamination of foods and crops can occur if crop drying is delayed and the moisture content is allowed to exceed critical values supporting mold growth during storage. Insect or rodent infestations can also potentially facilitate mold contamination of stored foods and feeds. Aflatoxins have been detected in milk, cheese, corn, peanuts, cottonseed, nuts, almonds, figs, spices, and a variety of other foods and feeds. Milk, eggs, and meat products are contaminated due to the ingestion of aflatoxin-contaminated feed by animals and birds (Finley et al., 1992). However, the commodities with the highest risk of aflatoxin contamination are corn, peanuts, and cottonseed.

Corn is probably the commodity of greatest concern worldwide, because it is grown in climates that are likely to have perennial contamination with aflatoxins. However, certain procedures used in the processing of corn help to reduce contamination of the resulting food product. For example, although aflatoxins are stable to moderately stable in most food processing operations, they are unstable in processes such as those used in making tortillas that employ alkaline conditions and oxidizing steps. Feeding of aflatoxin-contaminated corn and cottonseed meal to dairy cattle can result in aflatoxin M<sub>1</sub> contaminated-milk and milk products, including non-fat dry milk, cheese, and yogurt (Goldbatt, 1969; Heathcote and Hibbert, 1978).

Aflatoxins are one of the most potent toxic substances that occur naturally. Diet is the most important route through which humans and animals get exposed to aflatoxins. Extensive research on various animal species has revealed that aflatoxins, especially aflatoxin B<sub>1</sub>, are potential carcinogens (IARC, 1993). In humans, aflatoxins have been reported to cause hepatic cancer. A significant correlation between aflatoxin exposure and stunted

growth has been reported in children exposed to aflatoxin during neonatal stages. Moreover, since aflatoxins can cross the placental barrier, they can potentially lead to genetic defects in the fetus. Aflatoxins are immunogenic, teratogenic, and retard the growth in experimental animals. The adverse effects of aflatoxins on animals can be categorized into acute and chronic toxicity. Acute toxicity is caused when large doses of aflatoxins are ingested through feed. Chronic toxicity occurs following long-term exposure to moderate or low concentrations of aflatoxins. The symptoms in chronic aflatoxicosis include decrease in growth rate, lowered milk or egg production, and immunosuppression. The immuno suppressive effects of aflatoxins also predispose the animals to many secondary infections caused by other fungi, bacteria, and viruses (Robens and Richards, 1992; Mclean and Dutton, 1995).

#### 10.1.1.2. Ochratoxin A

Ochratoxin A (OTA) is a toxin naturally produced by *A. ochraceus*, its related species, and *Penicillium verrucosum*. Since these mold species are capable of growing on a wide variety of plants in different climatic conditions, contamination of food crops with OTA can occur worldwide. *A. ochraceus* is widely distributed on dried foods such as nuts, beans, dried fruits, and dried fish (Pitt and Hocking, 1997). OTA has also been reported on many other human foods, including cereals, cocoa, coffee, spices, and meat products (Jorgensen, 1998; MacDonald et al., 1999).

OTA is a nephrotoxic mycotoxin that primarily affects the kidneys in animals exposed to naturally occurring levels (Krogh, 1997). If the concentration of OTA is high, it can result in liver damage. In addition, OTA has also been linked to cancers of the human urinary tract (Ceovic and Miletic-Medved, 1996). Further, a few studies have demonstrated that OTA is genotoxic both in vivo and in vitro (Creppy et al., 1985; Bose and Sinha, 1994; Lebrun and Follmann, 2002). Research conducted on the possible effects of OTA on the immune system imply that OTA may affect both humoral and cell-mediated immunity (Singh et al., 1990; Stoev et al., 2000).

#### 10.1.1.3. Sterigmatocystin

Sterigmatocystin is a mycotoxin structurally related to aflatoxin (Rodricks, 1969), and is a metabolite of *A. versicolor*, *A. flavus*, *A. sydowi*, and *A. nidulans* (Davis, 1981; Terao, 1983). It is found to occur on grains, green coffee beans, maize, fruits, and grape fruit juice (Pande et al., 1990; Scudamore et al., 1996). Sterigmatocystin has been demonstrated to be acutely toxic, mutagenic, cytotoxic, and carcinogenic in in vitro and in vivo studies (Terao, 1983; Gopalakrishnan et al., 1992; Scudamore et al., 1996).

#### 10.1.1.4. $\alpha$ -Cyclopiazonic acid

Cyclopiazonic acid is a mycotoxin produced by several species of *Penicillium* and *Aspergillus*. It has been isolated from *P. cyclopium* (Holzapfel, 1968), and



was found to be a metabolite of *A. flavus* (Luk et al., 1977). It is commonly associated with maize and peanuts (Urano et al., 1992), millet (Rao and Hussain, 1985), tomato products (da Motta and Soares, 2001), and corn flakes (Aresta et al., 2003). Cyclopiazonic was found to be sub-acutely toxic to rats, swine, guinea pigs, dogs, and poultry, involving several vital organs (Voss et al., 1990; Smith et al., 1992). It is acutely toxic to rats and other test animals, resulting in severe gastrointestinal and neurological disorders (Nishie et al., 1985). Moreover, degenerative changes and necrosis have been observed in the digestive tract, liver, kidney, and heart (Norred et al., 1985).

### 10.1.2. PENICILLIUM

The literature on toxigenic penicillia is quite extensive, with almost 100 *Penicillium* species known to be toxigenic (Cole and Cox, 1981). The variety of different mycotoxins produced by *Penicillium* species is broader than that of any other fungal genus. The *Penicillium* mycotoxins of importance in fruits and vegetables include patulin, citrinin, cyclopiazonic acid, citreoviridin, ochratoxin A, and penicillic acid.

#### 10.1.2.1. Patulin

Patulin is a metabolite produced by a large number of fungi within several genera, including *Penicillium*, *Aspergillus*, and *Paecilomyces* species. Patulin has been detected on a variety of fruits such as pears, apples, apricots, peaches, grapes, and olives (Paster et al., 1995; Askar and Siliha, 1999; Arici, 2000; Abrunhosa et al., 2001; Moreau 2002). Patulin has been reported to be stable on dry cereals, and in apple and grape juices (Armentia et al., 2000; Moss and Long, 2002). Residues of patulin can cause safety issues in products such as juices derived from apples and citrus fruits. This is especially significant since apple juice intake can be considerably higher in infants and children (Verger et al., 1999; Beretta et al., 2000). The use of moldy fruits contaminated with *P. expansum* greatly increases the risk of patulin contamination in fruit juices. Patulin has been shown to be genotoxic (Pfeiffer et al., 1998), cytotoxic (Riley and Showker, 1991), immunotoxic (Bourdiol et al., 1990), and neurotoxic (Devaraj et al., 1982) in both in vivo and in vitro studies.

#### 10.1.2.2. Citrinin

Citrinin is a mycotoxin produced by *A. ochraceus*, *P. citrinum*, and related species (Frank, 1992). Citrinin can occur on grains such as rice, barley and maize, coconuts, apples, and related products. On grains, its presence is often found with ochratoxin A (Nishijima, 1984; Frank, 1992). Citrinin has been found to be a potent nephrotoxin in mice, rats, guinea pigs, rabbits, poultry, dogs, and pigs (Frank, 1992). Citrinin has also been shown to be embryotoxic,

teratogenic, and genotoxic in short-term tests. Benign renal adenomas were observed in rat fed with citrinin (Frank 1992).

#### 10.1.2.3. Citreoviridin

Citreoviridin is produced by *P. citreonigrum* (Pitt and Hocking, 1997), *P. ochrosalmoneum* (Wicklow and Cole, 1984), *P. citreoviride* (Morrissey and Vesonder, 1986), and *P. charlesii* (Cole and Cox, 1981). It occurs on maize, rice, and corn (Abramson, 1997). Citreoviridin has been implicated in acute cardiac beriberi associated with “yellow rice” condition in Japan (Ueno and Ueno, 1972). It has also been shown to cause paralysis of limbs, vomiting, convulsions, cardiovascular damage, and respiratory arrest in mice, cats, and dogs (Morrissey and Vesonder, 1986).

#### 10.1.2.4. Penicillic acid

Penicillic acid was first isolated from *P. puerile* and is now known to be produced by many *Penicillium* spp. as well as by members of the *A. ochraceus* group. It occurs on commercial maize and beans (Sanchis et al., 1988), peanuts, tree nuts, corn, and animal feeds (Jelinek et al., 1989). Penicillic acid has been reported to exert a variety of biological activities, including hepatotoxicity in experimental animals (Hayes et al., 1977; Dierickx and De Beer, 1984). It was also found to cause malignant, transplantable tumors in rats and mice (Dickens and Jones, 1961).

### 10.1.3. ALTERNARIA

*Alternaria* species are significant toxic contaminants of foods. *Alternaria* infects plants in the field, contaminating crops such as wheat, sorghum, and barley (Coulombe, 1991). *Alternaria* species also infect various fruits and vegetables, including apples, pears, citrus fruits, peppers, tomatoes, and potatoes and can cause spoilage of these fruits and vegetables in refrigerated conditions. *Alternaria alternata* produces a number of mycotoxins, including alternariol, alternariol monoethyl ether, altertoxin I, and tenuazonic acid (Scott, 2001). Natural occurrence of alternariol has been reported on various fruits such as tomatoes, peppers, olives, mandarins, melons, apples raspberries, and grains (Scott, 2001). The occurrence of low levels of alternariol in processed fruit products – apple juice, processed tomato produces, grape juice, and prune nectar – is of potential human health concern (Lau et al., 2003). Alternariol and alternariol monomethyl ether have been shown to be mutagenic (Schrader et al., 2001). Alternariol is also implicated in the development of squamous cell carcinoma. Moreover, it is linked to precancerous changes associated with esophageal mucosa in mice (Yekeler et al., 2001).

Alttoxins I is found on infected apples, wheat, and sorghum (Scott, 2001) and is a potent mutagen (Schrader et al., 2001).

## 10.2. DETECTION OF MYCOTOXIGENIC MOLDS

Accurate identification and enumeration of mycotoxigenic molds is essential for controlling these microorganisms in our food supply (Kurtzman et al., 1987; Beuchat and Cousin, 2001; Hoekstra et al., 2002; Samson et al., 2002). A proper sampling method is critical for the outcome of any mycological method. The sampling methods recommended by the International Commission on Microbiological Specifications of Foods (ICMSF) are used for food mycology (Pitt and Hocking, 1997; Tournas and Stack, 2001). Testing for the presence of mycotoxigenic molds is conducted under many different circumstances and in a variety of food matrices. Selection of the appropriate method depends on factors such as speed of the method, its accuracy, the skill level required to perform the assay, the cost and the intended use for the method. The methods basically fall into two major categories: those that are portable and applicable in the field (screening assays), and those that are conducted in analytical laboratories. In all cases, obtaining a representative sample is essential in order to ensure that results of the test can be correctly assigned to the sample being analyzed. Recently, progress has been made in the development of several rapid methods for the detection of molds on foods and feed. These rapid techniques can be grouped as chemical methods, immunological methods, and genetic methods (Gourama and Bullerman, 1995). The traditional identification procedures are based primarily on the recognition of the characteristics of the colony and micromorphology of the molds. Direct plating and dilution plating are the two main methods that have been used to detect as well as to enumerate molds on foods. However, the selection of agar media is important in the success of mold detection and enumeration on foods and feeds. During the last 20 years, food mycologists have been successful in developing a variety of selective and differential media for detecting and enumerating specific mold species.

One of the first selective media developed for detecting *A. flavus* was *Aspergillus* differential medium (ADM). In this medium, *A. flavus* and *A. parasiticus* are identified by their characteristic production of reverse orange-yellow color (Hocking, 1997; Hamsa and Ayres, 1997). Similarly, the *Aspergillus flavus-parasiticus* agar (AFPA) is used for the specific detection of *A. flavus* and *A. parasiticus* on corn, nuts, peanuts, seeds, and other commodities (Assante et al., 1981). In addition, *A. flavus* and *A. parasiticus* can be differentiated on Coconut cream agar, based on the difference in fluorescence of the colonies (Dyer and McCammon, 1994). Yet another medium called Sabouraud agar containing beta cyclodextrin was developed by Jaimez et al. (2003). This agar facilitates the simultaneous detection and enumeration of *A. flavus* and *A. parasiticus* under UV light. Likewise, PRYES

agar (pentachloronitrobenzene-rose bengal-yeast extract-sucrose agar) is a selective medium developed for the detection of ochratoxin A producing molds (Frisvad, 1983).

The chemical and biochemical methods for identifying molds are based on the detection and quantification of specific components of molds, such as chitin, ergosterol, fungal volatiles, and ATP. Chitin, a polymer of N-acetyl-D-glucosamine, is one of the major polysaccharides in mold spores and mycelia, and a good correlation exists between chitin or its breakdown compounds and mycelial growth on fruits and vegetables. The detection of chitin in foods and feeds indirectly implies fungal activity, even if the mold contaminants have been destroyed. A wide range of techniques, including colorimetry, chromatography (HPLC, GC), microscopy with fluorescent and non-fluorescent dyes and infrared spectroscopy, have been used to detect chitin (Ride and Drysdale, 1972; Wittenberg et al., 1989; Frey et al., 1994; Cousin, 1996). Ergosterol is the primary sterol in fungal membranes, and a positive correlation exists between ergosterol level and mold growth and subsequent toxin production. The analytical methods for ergosterol are based on direct saponification, extraction with hexane, and subsequent quantification using HPLC, TLC, LC, or spectrophotometric techniques (UV, infrared) (Seitz, et al., 1977; Zill, et al., 1988; Frey et al., 1994; Bermingham et al., 1995; Gourama and Bullerman, 1995).

There are a few disadvantages associated with the use of the conventional detection techniques. They are time-consuming, labor-intensive, require special facilities and mycological expertise (Lacey et al., 1980; Jarvis et al., 1983). Furthermore, some of these methods are occasionally inaccurate yielding results that are not reproducible. This led to the development of several serological and molecular methods that are rapid, sensitive, and suitable for the detection of toxigenic molds. These methods could be applied both in the field and after harvesting in order to obtain real-time monitoring data on contamination, thereby assisting in food safety assessment. Table 10.2 summarizes the various methods available for the detection of *Aspergillus*, *Penicillium*, and *Alternaria* on fruits and vegetables.

Methods involving immunological assays for mold detection are based on the binding of fungal antigens and their corresponding antibodies. A variety of different sources of fungal material have been used as antigens, which are heat resistant, and thus persist on foods and feeds even after the destruction of mold species. The different fungal materials used as antigens include direct fungal material such as mycelium fragments, spore suspension, mixtures of spores and mycelia, and the indirect fungal material such as surface washes from the growth media and extracellular polysaccharide (Notermans et al., 1986; Bhatnagar et al., 1989; Cousin et al., 1990; Kamphuis et al., 1992; Tsai and Cousin, 1993; Pestka et al., 1995; Yong and Cousin, 2001). Direct and indirect immunosorbent techniques have been used to detect a wide range of molds (Lin et al., 1986; Lin and Cousin, 1987; Van der Horst et al., 1992; Gourama and Bullerman, 1995). Among the immunochemical methods, ELISA is the most widely used. Most of the ELISA methods for detecting

TABLE 10.2 Detection of mycotoxigenic *Aspergillus* spp., *Penicillium* spp. and *Alternaria* spp. in fruits and vegetables.

Species	Detection method	References
<i>Aspergillus</i>	<i>Selective media</i>	
	Sabouraud agar with 0.3% cyclodextrin	Jaimez et al., 2003
	Coconut cream agar	Dyer and McCammon, 1994
	<i>Aspergillus flavus</i> and <i>A. parasiticus</i> agar (AFPA)	Pitt and Hocking, 1997
	<i>Immunoassays</i>	
	EIA-Europium-linked time-resolved fluoroimmunoassay	Paugam et al., 1998
	ELISA	Yong and Cousin, 2001; Park et al., 2003; Tsai and Yu, 1999
	Indirect noncompetitive ELISA	Anand and Rati, 2006
	Patorex <i>Aspergillus</i> latex agglutination test	Ansorg et al., 1997
	<i>Molecular methods</i>	
	PCR	Shapira et al., 1996; Farber et al., 1997; Reddy et al., 1993; Chen et al., 2002; Dao et al., 2005
	Multiplex PCR	Sartori et al., 2006
	AFLP	Niessen et al., 2005
		Perrone et al., 2006; Schmidt et al., 2003
		Niessen et al., 2005
	RAPD	Brandt et al., 1998; Fungaro et al., 2004
	Taqman detection assay	Brandt et al., 1998
	Real time PCR	Atoui et al., 2007
		Mule et al., 2004b;
	Quantitative PCR	Perrone et al., 2006
	RFLP	Somashekar et al., 2004
	DNA based plate hybridization assay	Fletcher et al., 1998
	PCR-based line probe assay	Martin et al., 2000
	<i>Spectroscopy</i>	
	FITR-PAS	Gordon et al., 1997; Gordon et al., 1999
	TIRS	Gordon et al., 1999
	TIRES	Gordon et al., 1999
	Novel delayed luminescence spectra	Chen et al., 2005
	PCR-EIA	Aguirre et al., 2004
	Reverse line blot hybridization	Playford et al., 2006
	<i>Chromatography</i>	
	Micellar Electrokinetic Capillary Chromatography	Martin et al., 2005
<i>Alternaria</i>	Double-sandwich ELISA	Lin et al., 1986
	Double immunostaining	Green et al., 2005
	PCR	Zur et al., 1999

TABLE 10.2—(Cont'd)

Species	Detection method	References
Penicillium	<i>Immunoassays</i>	
	ELISA	Kamphuis et al., 1992; Li et al., 2000; Park et al., 2003
	Double immunostaining	Green et al., 2005
	<i>Molecular methods</i>	
	PCR	Pedersen et al., 1997
	AFLP	Marek et al., 2004
	RAPD	Niessen et al., 2005
	Real time PCR	Geisen et al., 2004
	Quantitative PCR	Haugland et al., 2004
	Electronic Nose	Keshri et al., 1998
	MECC	Martin et al., 2005

molds have focused on the development of assays using antigens from a specific fungal species. Although such immunological assays have proven useful to estimate total fungal biomass, it has been reported to be difficult to raise antiserum that will specifically detect a single species of mold in a mixed culture (Notermans et al., 1986; Lin and Cousin, 1987; Banks et al., 1994). However, the advent of the hybridoma technology facilitated mycologists to raise and select hybridoma cell lines that secrete monoclonal antibodies to fungi with adequate specificity. Direct and indirect ELISA have been developed to detect the presence of *Penicillium* and *Aspergillus* species in fruit juices, spices, cereals, and foods (Notermans et al., 1986) and for the detection of *Alternaria* in tomato puree (Lin et al., 1986). Warnock (1973) developed polyclonal antibodies against mycelia obtained from *Alternaria*, *Aspergillus*, and *Penicillium* for the detection of these molds on grains and feed.

Nucleic acid-based detection methods RAPD (Niessen et al., 2005), AFLP (Niessen et al., 2005), RFLP (Niessen et al., 2005), real-time quantitative PCR (Niessen et al., 2005), multiplex PCR (Yang et al., 2004), and PCR-ELISA (Grimm and Geisen, 1998) have been developed for the detection of major mycotoxigenic species associated with fruits and vegetables. A number of PCR-based methods have been developed for the identification of mycotoxin biosynthetic genes in different fungal genera, together with assays developed, using other genes or random amplification of polymorphic DNA for the identification of specific toxigenic fungi (Dao et al., 2005). In addition, reverse transcription (RT)-PCR, competitive PCR, and real-time quantitative PCR have also been developed for this purpose. PCR identification of consensus sequences obtained from specific phylogenetic information can be inferred for all taxonomic groups of mycotoxigenic fungi. A few of these PCR are based on exploiting interspecies differences between sequences of nuclear ribosomal intergenic spacer DNA (IGS rDNA), beta-tubulin, calmodulin, elongation

factor, and AFLP analysis (Schmidt et al., 2003; Mulè et al., 2004a,b). Similarly, other researchers have designed oligonucleotide primers for the PCR amplification of genes involved in the biosynthesis of some mycotoxins (Seo et al., 2001; Sidhu, 2002). These molecular markers can be successfully applied for the detection of toxigenic fungal species on fruits and vegetables.

As the complete genome sequences of mycotoxigenic fungi become available, microarrays using specific gene sequences are being developed (Desjardins and Bhatnagar, 2003). Microarray technology can be used for detecting toxigenic species belonging to the same genus or different genera. This is especially important since by large it is not a single species but a complex of species found on fruits and vegetables. In this context, in order to prevent the toxicological risk, it is critical that the method used is able to detect toxigenic fungal populations in a short time frame. Molecular markers employed for PCR-based detection can be implemented in DNA biochips. DNA microarrays consist of ordered sets of DNA molecules of known sequence and specificity fixed in spots to small solid surfaces. A sample containing previously labeled DNA or RNA is applied to the surface, and complementary sequences can bind to the arrayed DNA probes. The hybridization results are imaged and can be either quantitatively or qualitatively analyzed. A new type of low-complexity oligonucleotide microarray concept applied to the identification of toxigenic fungi has been proposed (Guerniou et al., 2004). Apibio biochips are used in many applications mainly for monitoring and testing gene expression, genotyping analysis (Bufflier et al., 2003; Guerniou et al., 2004), and protein identification (Perrin et al., 2003). OLISA<sup>TM</sup> (OLigo Sorbent Arrays) are microarrays of up to 17 probes, including internal controls, arrayed on a 96-well microtiter plate. Short oligonucleotide probes are designed and optimized to hybridize specifically to any single nucleotide polymorphism of an amplified DNA target. This microarray format is well adapted to detect different toxigenic fungi simultaneously in food samples. Toxigenic fungal genomic DNA extracted from food can be subjected to multiplex PCR to generate biotin-labeled fragments and detected on OLISA microarray by colorimetry (Bufflier et al., 2003). The wells are then imaged and analyzed using an automated system. The associated software allows an automatic analysis of images and provides a final report of results. This enables the detection of all toxigenic fungi strains in one step.

During exponential growth, many fungi release low molecular weight, volatile organic compounds as products of secondary metabolism. Considering that the composition of these volatile organic compounds remains qualitatively stable over a range of growth media and conditions, an attractive and promising method for analyzing mold contamination based on the use of multisensing systems technology (electronic nose) (Gardner and Bartlett, 1994; Taurino et al., 2003) has been proposed, allowing an accurate evaluation of mold contamination in foods in minutes (Olsson et al., 2000; Magan and Evans, 2001). The principle behind the functioning of electronic noses is similar to that of the human nose: several reactions take place between one or more volatile components and an array of sensors. This relatively rapid

reaction involves the adsorption/desorption of volatiles on the surface of the sensors producing a signal, which depends on the transduction method of the sensors. The responses obtained from the sensor are analyzed in a subsequent data processing step in order to perform the required qualitative and quantitative analysis of the ambient examination. Currently, the major market for electronic noses is the food industry. Applications of electronic noses in the food industry include quality assessment in food production, inspection of food quality by odor, and inspection for spoilage by molds. Specific fungal volatile markers can be used by electronic nose to detect mycotoxigenic fungi on fruits and vegetables.

A concept similar to electronic nose, but applicable for detecting molds in liquids such as fruits juices, called the “electronic tongue”, has been described (Toko and Fukusaka, 1997). Similar to the electronic nose, the basic principle behind an electronic tongue is to combine signals from non-specific and overlapping bio- or chemical sensors with pattern recognition routines. The sensor arrays used for liquid sensing are based on electrochemical methods such as potentiometry and voltammetry (Soderstrom et al., 2003). The electronic tongue is described based on an array of chalcogenide glass sensors, including conventional electrodes such as chloride, sodium, and potassium selective sensors, combined with a pattern recognition routine. Taste sensors based on lipid/polymer membranes on a multichannel electrode are used (Imamura et al., 1996; Toko and Fukusaka, 1997).

Recent technological advances in Fourier transform infrared (FTIR) spectroscopy have facilitated infrared analyses of biological materials in their solid states. FTIR spectroscopy is exceptionally specific and provides quantitative and qualitative information, especially in the mid-infrared absorption range where most biological materials have distinctive spectra (McDonald, 1986). Because of its inherent sensitivity to the near-surface region of the sample, FTIR-Photoacoustic Spectroscopy (FTIR-PAS) is an excellent sensor for fungal detection on the surface of corn kernels (Greene and Gordon, 1992; Greene et al., 1992). In spite of the high sensitivity of the FTIR-PAS technique, its current application is limited by the design of commercially available photoacoustic detectors to analysis of a single corn kernel in a small sealed sample cell. Transient Infrared Spectroscopy (TIRS) is a potential new technique that overcomes the limitations of photoacoustics and midrange infrared methods by permitting non-contact analyses of a flat bed of solid materials moving at conveyor belt speed (Jones and McClelland, 1990a,b, 1993). TIRS works by heating or cooling the surface of moving material to create a thin non-opaque surface layer that is ideal for infrared analysis. In TIRS, an optically thin surface layer is optionally either rapidly heated or cooled for an instant before the sample passes through the field of view of an infrared spectrophotometer. When the surface layer is made hotter than the bulk of the kernel, the technique is called a transient infrared emission spectroscopy (TIRES). When the surface is made colder than the bulk, the technique is called transient infrared transmission spectroscopy (TIRTS). Both techniques offer the possibility for online evaluation of feed for the possible presence of fungal infection.



### 10.3. FUTURE CHALLENGES AND MILESTONES

Sensitive and reproducible detection and quantitation of molds present on complex matrices such as foods presents certain challenges in the development of assays. Predominantly, these include ensuring that the sample being tested is representative of the larger lot, and that the assays are sensitive and accurate for their intended purpose. Rapid detection of molds is an area where emerging technologies may find an application. It is technically possible to develop antibody-based screening assays for multiple molds using array formats similar to those described for specific mold species (Rowe-Taitt et al., 2000). The continued compactness of analytical instrumentation also lends the prospectus for hand-held devices performing the same functions as their bench top counter parts. The developments in antibodies and antibody fragments through recombinant technologies represent a potential approach for improving immunoassays (Zhou et al., 1996; Yuan et al., 2000).

The emerging technologies for mold detection are at various stages in the evolution to practically applicable analytical tools. Although some of the technologies have progressed enough for field applications, many still face the challenge of transition from proof-of-concept assays using molds in pure cultures to application on food samples. However, a few other methods have been shown to be suitable for use in food samples, although they need to be simplified for easy application. Despite these obstacles, innovative detection technologies continue to progress, enabling specific and sensitive detection of molds, thereby aiding in the control of food-borne mycotoxicosis.

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# Detection and Determination of Ochratoxin A in Grape Products

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## ABSTRACT

Ochratoxin A is a mycotoxin often found in grapes and grape products. Since it was first reported in wines approximately 10 years ago, several developments have been reported in its analysis from grapes and their derived products. Methods for qualitative, semi-quantitative and quantitative analyses have been developed in different formats. Most of these methods involve five main stages: sampling, sample preparation, extraction, clean-up and quantification. Sampling and sample preparation are two of the most critical stages, since the material portion taken to the laboratory for analysis should represent as accurately as possible the whole lot. The design of subsequent stages will be very dependent on the measurement (separation, detection and quantification). The extraction stage aims at solubilizing the mycotoxin in an appropriate solvent, and – depending on the measurement method – various clean-up strategies are available. Highly specific and selective measurement will require less extensive clean-up and vice versa. Most of the methods rely on the general guidelines for sampling, an extraction with an aqueous-based solvent, clean-up with immunoaffinity columns and quantification by a chromatographic method coupled with fluorescence or mass detection. In this chapter, the recent developments in ochratoxin A detection are presented and the most representative methods for the analyses of grapes, dried vine fruits and wine are presented and discussed.

## 11.1. INTRODUCTION

Ochratoxin A (OTA) is a secondary metabolite produced by strains of a few filamentous fungi species. Because of its toxic properties and the fact that it is detected in food, this metabolite is known as a mycotoxin. OTA is an important nephrotoxic mycotoxin having also immunosuppressive, teratogenic, genotoxic and carcinogenic effects and was classified by the International Agency for Research on Cancer as a carcinogenic metabolite belonging to

group 2B. OTA was named after the fungus from which it was first isolated and identified – *Aspergillus ochraceus* (van der Merwe et al., 1965).

The first mycotoxin reported in fruits was patulin. It is a small, unsaturated heterocyclic lactone, produced by species of *Penicillium*, *Aspergillus*, *Byssoschlamys* and *Paecilomyces*. *Penicillium expansum*, the causing agent of the blue mold rot of apples, pears and other fruits, is the most known producer of patulin. It is also associated with the contamination of grape products, following the use of moldy grapes (Scott et al., 1997; Drusch and Ragab, 2003). *Byssoschlamys* spp. are noteworthy as they may produce heat-resistant spores which survive food processing with the potential to produce patulin in heat-treated products such as canned food (Rice et al., 1977). The significance of this mycotoxin has been increased after the recent statutory EU regulations in fruit products (Venâncio and Paterson, 2007).

While being detected in grape juices and grape musts, patulin was not reported in grapes or dried vine fruits (Drusch and Ragab, 2003), although *P. expansum* has frequently been isolated from these commodities (Abrunhosa et al., 2001). Patulin has not yet been detected in wine (Ough and Corison, 1980), mainly due to the degradation of patulin by *Sacharomyces cerevisiae* during anaerobic fermentation (Moss and Long, 2002). Other *Penicillium* toxins have been reported in grape products. For instance, the clavine alkaloid isofumigaclavine A (roquefortine A), produced by *Penicillium roqueforti* and other species, was formed in homemade wine contaminated with *P. roqueforti* or *P. crustosum* before fermentation (Möller et al., 1997).

There are no reports on the presence of aflatoxins in grape, grape juices and grape musts. These mycotoxins have been screened in dried vine fruits by several authors, but just in one study was aflatoxin B<sub>1</sub> detected, although at a rather low incidence (Drusch and Ragab, 2003). Although the presence of aflatoxins have also been investigated in wines and some very sensitive methods for their extraction and quantification from wine have been developed (Takahashi, 1974, 1977), these mycotoxins were not detected.

The first reference to the presence of a toxic fungal metabolite in wine was made in the late 1980s, when a research group from Germany reported on the presence of *Trichothecium roseum* metabolites in white wines, namely trichothecin, trichothecolon and rosenolactone (Schwenk et al., 1989) although at concentrations that did not present a hazard for public health. The same authors mentioned, however, that trichothecin, a member of the well-known trichothecene family, is toxic to yeasts, inhibiting alcoholic fermentation. Recently, alternariol, a mycotoxin produced by *Alternaria* species, has been reported in one sample of red wine by Lau and co-workers (2003).

OTA was first detected in wine by Zimmerli and Dick (1995). While performing an epidemiologic study of the Swiss population, these researchers found OTA in biological samples (blood, serum and human milk). When assessing the exposure of this population to OTA, the presence of the mycotoxin was detected in wine samples. Since the findings of Zimmerli and Dick, several surveys of different grape products have been carried out worldwide. OTA was detected in table and wine grapes, dried vine fruits, grape juices and

musts, vinegar and wine (MacDonald et al., 1999; Otteneder and Majerus, 2000; Markaki et al., 2001; Sage et al., 2002; Battilani and Pietri, 2004; Belli et al., 2004; Serra et al., 2004).

Despite all these reports on the presence of mycotoxins in grapes and grape products, just three of the mycotoxins were found to be of concern to the public health, namely patulin in non-fermented grape products, aflatoxins in dried vine fruits, and OTA in all the grape products studied. This chapter deals with the detection and determination of OTA in grape products.

## 11.2. DETECTION OF OCHRATOXIN A IN GRAPES AND WINE

The analytical methodology for the detection of contaminants in foods involves a sequence of five steps: (i) sampling; (ii) sample preparation; (iii) extraction; (iv) clean-up; and (v) quantification.

### 11.2.1. SAMPLING AND SAMPLE PREPARATION

The sampling stage is one of the most critical steps for a precise analytical procedure. This is even more important in the case of mycotoxins in particulate materials, since it is well known that the occurrence of mycotoxins is very heterogeneous. Sampling plays a crucial role in the analyses of whole grapes or dried vine fruits. However, in wine, grape juices and grape musts, sampling is more straightforward due to the liquid nature of these products.

The estimation of OTA level in a bulk lot of grape products is based on the concentration of incremental samples taken from the lot. In a regulatory environment, based on the measured concentration of these samples, a decision will be made on the acceptability of the lot. Obviously, if the sample concentration does not accurately reflect the lot concentration then misclassification may occur. This is why sampling is one of the most critical stages. A considerable work has been done on sampling plans, and in the EU there are regulations for the sampling method for the official control of OTA levels in dried vine fruits, grape juice, grape must and wine (Commission Regulation, 401/2006).

A sampling plan includes (i) the collection of material from single places in the lot or sub-lot, i.e. an incremental sample; (ii) the combining and homogenization of all the incremental samples, i.e. an aggregate sample; and (iii) the removal of a portion of the combined material for analytical evaluation, i.e. a laboratory sample.

The sampling plan approved by the EU for dried vine fruits established the collection of up to 100 incremental samples of 100 g to yield an

aggregate sample of 10 kg for each lot with 15–30 tons of dried vine fruits. Lots of lower size require fewer samples, and of a smaller size. For grape must, grape juice or wine, depending on the size and form of commercialization of the lot (e.g., bottle, bulk), incremental samples are of at least 100 g, resulting in an aggregate sample of at least about 1 kg. From the aggregate sample, laboratory samples are withdrawn for OTA quantification. Sampling and sample preparation are inevitable, difficult and time consuming.

### 11.2.2. EXTRACTION

Determination of mycotoxins from any food commodity requires a step for the solubilization of the mycotoxin with an appropriate solvent. In most of the cases, this is done with an immiscible solvent (i.e., a solid–liquid or a liquid–liquid extraction step); however, in some cases, with liquid foods it is possible to omit the liquid–liquid extraction step, replacing it by a dilution with an appropriate aqueous solvent. When the food sample is a solid matrix, the solid–liquid extraction is usually carried out in a blender over a defined period of time. This is a difficult step, since it is not possible to know the quantitative recovery of the mycotoxin as the actual level in the sample is unknown, i.e. it is not possible to calculate the relative amount of mycotoxin extracted or solubilized by the solvent since the amount of mycotoxin initially present is unknown. It is often said that the relative amount of mycotoxin extracted by the solvent (the recovery) could be determined by spiking the sample with a known amount of mycotoxin. However, spiking of the sample will not give a representative contaminated sample. Spiking does not seem the best way to determine the recovery of an extraction step, but rather as being an indication of any losses at subsequent stages of the assay after extraction has been done (Gilbert, 1999). On the other hand, when dealing with liquid samples, it is possible to spike the sample and quantify the recovery.

#### 11.2.2.1. Grapes and dried vine fruits

The determination of OTA from grapes or dried vine fruits should be different from the determination of the mycotoxin from grape juice or wine, due to the physical nature of the sample. The first ones need additional extraction, usually with mechanical shaking or blending.

Since the distribution of OTA in grapes or dried vine fruits is probably not homogeneous, an initial extraction process involving the use of a mechanical shaker or a blender is necessary to assure homogenization of the sample. There are two methods published for this determination, both use high-speed blenders and solvent systems to hydrate and solubilize OTA. Details on both methods are provided in Table 11.1.

TABLE 11.1 Comparison of methodologies for the determination of ochratoxin A from grapes and dried vine fruits.

Method	
Serra et al. (2004)	MacDonald et al. (1999)
1st – blend grapes for 10 min at high speed	1st – mix 25 g of dried vine fruits, with 20 ml of water
2nd – take 50 g of blended grapes	2nd – add 50 ml of methanol and 5 ml of orthophosphoric acid
3rd – add an aqueous solution of 5% NaHCO <sub>3</sub> /1% PEG 8000 till a final volume of 150 ml	3rd – homogenize 2 min in an Ultra-Turax
4th – centrifuge at 8500 rpm for 20 min, at 4°C	4th – filter through a Whatman No. 1 paper
5th – filter through a 1.6 µm pore size filter	5th – take 12.5 ml of filtrate and dilute to 100 ml with PBS
6th – prepare the IAC according to the manufacturer and pass 20 ml of the filtrate through it	6th – prepare the IAC according to the manufacturer and pass 50 ml of the diluted filtrate through it
7th – wash the IAC according to the manufacturer and elute OTA with 2 ml methanol	7th – wash the IAC according to the manufacturer and elute OTA with 2 ml of 2% acetic acid in methanol
8th – dry the eluate and re-suspend the sample in HPLC mobile phase	8th – dilute the eluate in an equal volume of 2% acetic acid
9th – inject sample in the HPLC	9th – inject sample in the HPLC

11.2.2.2. Wine, grape juice and musts

The determination of OTA from wine, grape must or grape juice was initially done by means of an extraction with an immiscible solvent, such as acidified chloroform or toluene (Table 11.2). The use of acidified chloroform is a very common way to determine OTA in foods, since OTA binds to proteins and this solvent breaks the bond (Valenta, 1998). The use of organic solvent is useful when the subsequent separation and quantification is by thin layer chromatography (TLC). However, when further sample clean-up is needed, and the use of immunoaffinity columns (IAC) is required, the organic solvent must be replaced by an aqueous one, as is the case with the more recent methodologies.

In the case of wine and grape juices, to avoid this additional step of solvent change, it is possible to omit the liquid–liquid extraction step, replacing it by dilution with a bicarbonate solution or a phosphate buffer solution (Table 11.2). In grape products, especially red grape products, the presence of a substantial amount of coloured compounds (mainly tannins) interferes with the interaction of the antibodies of IACs and the mycotoxin. To reduce this interaction, Visconti and co-workers (1999) used polyethylene glycol (PEG) in their dilution solution. These researchers recommend the dilution of the grape juice or wine sample with an aqueous solution containing 5 percent of bicarbonate and 1 percent of PEG (molecular weight of 8000 Da) (Table 11.3).



TABLE 11.2 Methodologies for the determination of ochratoxin A in grape products.

Commodity	Extraction	Clean-up <sup>1</sup>	Separation	Detection <sup>2</sup>	Confirmation	Reference
Grapes	5% NaHCO <sub>3</sub> + 1% PEG 8000	IAC	HPLC	FD	Derivatisation with BF <sub>3</sub> in methanol	Serra et al. (2004)
dried vine fruits			HPLC	FD	–	MacDonald et al. (1999)
Wine	acidified chloroform	IAC	HPLC	FD after post-column derivatisation	Derivatisation with methanol and HCl	Zimmerli and Dick (1996)
Wine	Toluene	SPE	HPLC	FD	Standard addition	Festas et al. (2000)
Wine	5% NaHCO <sub>3</sub> + 1% PEG 8000	IAC	HPLC	FD	Derivatisation with BF <sub>3</sub> in methanol	Visconti et al. (1999)
Wine	Phosphate buffer system	IAC	HPLC	FD	–	Otteneder and Majerus (2000)

1 – clean-up: SPE = solid phase extraction; IAC = immunoaffinity column

2 – FD = fluorescence detector

**TABLE 11.3** Recommended methodology for the determination of ochratoxin A from wine (Visconti et al., 1999).

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**Methodology steps**

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- 1st – degas sparkling wines, by stirring or sonication, for 15–20 min;
  - 2nd – take 10 ml of degassed wine and dilute it with an equal volume of a dilution solution (1% PEG 8000 and 5% NaHCO<sub>3</sub>);
  - 3rd – filter through a 1.6 µm pore size filter;
  - 4th – prepare the IAC according to the manufacturer and pass 10 ml (equivalent to 5 ml of wine) of the filtrate through it;
  - 5th – wash the IAC according to the manufacturer and elute OTA with 2 ml methanol;
  - 6th – dry the eluate and re-suspend the sample in HPLC mobile phase;
  - 7th – inject sample in the HPLC.
- 

### 11.2.3. CLEAN-UP

The clean-up stage of the analytical procedure involves the separation of the mycotoxin from other co-extracted compounds. The design of this stage is highly dependent on the subsequent quantification stage. Qualitative assay will require less or no clean-up, while quantitative ones will probably require more extensive clean-up. The specificity and sensitivity of the quantification will determine the required extent of the clean-up stage. Highly sensitive and specific immunoassays may not need a clean-up stage (e.g., enzyme linked immunosorbent assay), whereas methods with occasionally poor resolution (e.g., TLC) will require extensive clean-up.

In the analyses of grapes and grape products, the use of clean-up is recommended. Clean-up may be achieved by solid phase extraction (SPE). SPE is a technique used to adsorb selectively an analyte from a liquid solution into a solid matrix. Besides cleaning, SPE may be used for sample concentration and/or for solvent change. The general procedure is to load a solution onto the SPE phase, wash away undesired components, and then wash off the desired analytes with another solvent into a collection tube. This method is time and labour consuming.

More recently, the use of immunoaffinity assays have decreased the time of analysis. Based on antigen–antibody reactions, these assays have been developed in different modes, for qualitative, semi-quantitative or quantitative analyses (Visconti and De Girolamo, 2005). Although immunochemical assays have become popular, they require stable antibodies, are expensive and have limited ‘reusability’.

For grape products analysis, the available clean-up procedures include the following:

- (i) SPE cartridges for OTA determination from wine (Festas et al., 2000);
- (ii) Lateral Flow devices (LFD), based on carrier membrane with immobilized antibodies, were initially developed for detection of OTA-producing fungal strains, and later adapted for screening of OTA in food commodities, such as wine;

- (iii) IAC for OTA determination from wine (Visconti et al., 1999), and later adopted as the European standard for OTA determination from wine and beer.

Other clean-up strategies have been applied to the determination of OTA from grape products, but these have not yet proven to be at a commercial stage of exploitation. Molecular imprinted polymers (MIP) (Maier et al., 2004), array biosensors (Ngundi et al., 2005) or liquid-phase micro-extraction (LPME) (González-Peñas et al., 2004) are a few examples of these recent developments.

#### 11.2.4. QUANTIFICATION

After clean-up and before final measurement (quantification), the mycotoxin must be free of all interferences. Separation of the mycotoxin is usually achieved by a chromatographic method, either TLC, gas chromatography (GC) or liquid chromatography (LC). Due to the non-volatile nature of OTA, samples are separated by either TLC or LC and quantified by fluorescence or mass spectrometry (MS).

The use of the fluorescence detection (FD) is very common for the quantification of OTA, but the MS detection is increasing its importance, since it is a way of quantifying and confirming the identity of the mycotoxin. When the FD is used, an additional confirmation step may be needed to assure the identity of OTA. Confirmation is usually done by derivatization with  $\text{BF}_3$  in methanol or with methanol in concentrated hydrochloric acid. Both these procedures yield the methyl ester derivative of OTA. Recently, a new method has been proposed, using an LC-FD with an alkaline eluent, allowing the detection of OTA by direct injection of wine samples at the same level as the LC-MS analysis (0.05 ng/ml). According to the authors (Dall'Asta et al., 2004), the method is straightforward, quicker, reproducible, inexpensive and gives a comparable detection limit as the much more expensive LC-MS.

##### 11.2.4.1. Grapes and dried vine fruits

The literature describes two methodologies for the quantification of OTA from grapes and dried vine fruits (Tables 11.1 and 11.2). The performance of both methods was evaluated by Serra and co-workers (2004) using grapes spiked with an *Aspergillus carbonarius* strain. Both methods proved to be very precise and comparable over a wide range of OTA concentrations (from 0.07 to 37.6  $\mu\text{g/kg}$ ) with recoveries above 70 percent. The main difference is that one of the methodologies (MacDonald et al., 1999) uses an organic solvent in the extraction stage, while the other one uses an aqueous solvent system.

##### 11.2.4.2. Wine, grape must and juice

For wine, grape must or juice, several methodologies have been proposed, most of them based on a common clean-up stage with an IAC. Some minor

differences are proposed for the extraction stage, and the main differences are observed in the separation and quantification stage. The most popular method is the one of Visconti and co-workers (1999). In a recent interlaboratorial study (Ratola et al., 2006), with the participation of 24 laboratories from 17 countries, with no restrictions in terms of analytical methodology to employ, 75 percent of the laboratories have used the method of Visconti and co-workers (1999). The remaining laboratories have used alternative extraction, clean-up or quantification approaches. In terms of performance, all the methods used for OTA determination in wines were reproducible.

### 11.3. SAFETY AND CONCLUSIONS

Ochratoxin A is a toxic compound that needs to be manipulated with care and with appropriate safety precautions. Decontamination procedures for mycotoxins in laboratory wastes have been reported by the International Agency for Research on Cancer (Castegnaro et al., 1991) and should be employed throughout analytical work.

The development of an analytical method for the quantification of OTA from grape products is the result of a balance between the performances of each individual stage. The first stage of sampling is the most critical, since a less representative sample will lead to a deficient evaluation of the quality of an entire lot. In some countries, such as those in the EU, there are guidelines for sampling that hopefully will circumvent this risk. After sampling and sample preparation, the most important stage is measurement. The more specific this stage is, the fewer clean-up and separation stages will be required.

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# Detection and Determination of Patulin in Fruits and Fruit Products

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## ABSTRACT

Patulin (PAT) is a toxic metabolite produced by several species of the genera *Penicillium* and *Aspergillus*, and it has been identified in many moldy fruits, vegetables, cereals, and other foods. The most commonly occurring patulin producing species is *Penicillium expansum*.

Patulin has been found as a natural contaminant of processed fruits, most notably apple juices and other apple-based products, and it has been suggested to be indicative of the quality of the fruit used for production. Some reviews have been focused on patulin toxicity, although its carcinogenic effects have not been fully elucidated. Regulatory limits have been established for PAT to minimize human and animal exposure and this limit has provided an incentive for the development of faster and more specific analytical methods with lower detection limits. Analytical methods for identifying and quantifying patulin in fruits and fruits juice are widely reviewed and discussed.

## 12.1. INTRODUCTION

Patulin (PAT) is a toxic metabolite produced by certain species of *Penicillium*, *Aspergillus*, and *Byssoschlamys*. It has been identified in many moldy fruits, vegetables, cereals, and other foods; however, the major sources of contamination are apples and apple-based products (Moss, 1998; Puschner, 2002; Gilbert and Pohland, 2003). The most commonly occurring PAT producing species is *Penicillium expansum*, producing the mycotoxin in apples, pears, cherries, and other fruits. PAT contamination is primarily associated with areas of decomposing tissue, and can penetrate up to approximately 1 cm into the surrounding healthy tissue (Taniwaki et al., 1992). The presence and the extent of PAT contamination in processed apple products can serve as a marker for the quality of the fruit used



for processing and the quality of the product. Some reviews focus on PAT toxicity, although its carcinogenic effects have not been fully elucidated (Atkins and Norman, 1998; Moss, 1998; Askar, 1999; Puschner, 2002). The International Agency for Research on Cancer (IARC, 1986) concluded that no evaluation could be made of the carcinogenicity of PAT to humans and that there was inadequate evidence in experimental animals.

The Joint FAO/WHO Expert Committee on Food Additives and Contaminants (JECFA) established a provisional maximum daily intake of 0.4  $\mu\text{g/kg}$  body weight/day. This intake is based on the calculated and not on the observed effect level (NOEL) (WHO, 1995).

Chemically, PAT is an  $\alpha$ ,  $\beta$ -unsaturated lactone composed of 4-hydroxy-4H-furo[3,2-c]pyran-2[6H]one (Fig. 12.1).

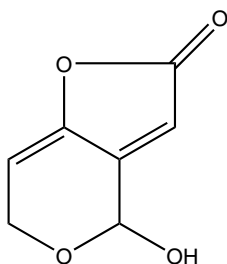


FIGURE 12.1 Chemical structure of patulin.

PAT forms colorless crystals with a molecular weight of 154 and melting point of 111°C. It is gradually destroyed during storage in the presence of sulfites, sulfhydryl groups, and ascorbic acid (McKenzie et al., 1997).

Many countries regulate PAT in juice at levels ranging from 5–10  $\mu\text{g/kg}$ , e.g. South Africa and the United Kingdom has recommended PAT concentration of <50  $\mu\text{g/kg}$  for apple products intended for human consumption (FAO, 2004). The European Union established a limit of 10  $\mu\text{g/kg}$  for baby food and infant formulae (EC, 2003).

## 12.2. PATULIN ANALYSIS

The established limit for PAT has provided an incentive for the development of faster and more specific analytical methods with lower detection limits. A comprehensive review of the development of thin layer, gas, and liquid chromatographic methods for the detection and confirmation of PAT has been published by Shephard and Leggott (2000).

A variety of methods have been developed for extraction, detection, quantification, and confirmation of PAT in food products. Traditional chromatographic techniques such as thin layer chromatography (TLC), gas chromatography (GC), and liquid chromatography (LC) are widely employed for separation and quantification.

Analytical procedures involve three basic steps, extraction, purification/separation, and identification/quantification. In most of the currently methods

used for the analysis of fruit juices as well as other products, including the official method of the International Association of Official Analytical Chemists (AOAC) (1995, 2000), a liquid–liquid extraction and solid-phase extraction (SPE) are used. Several analytical methods have been proposed, mainly concerning TLC, GC, and LC with UV detection. Recently, mass spectrometry (MS) has been associated with both high performance liquid chromatography (HPLC) and GC, allowing for a conclusive confirmation of the toxin. MS–MS methods provide additional selectivity and increased sensitivity.

Few problems from coextractants are experienced with apple juice, but concentrated products are more troublesome. A compound identified as 5-hydroxymethylfurfural (HMF) was found to have chromatographic characteristics similar to those of PAT (WARE, 1975). Gökmen and Acar (1999) described a rapid and economical method for the simultaneous determination of HMF and PAT in apple juice.

### 12.2.1. EXTRACTION AND CLEANUP

The solvent used to extract PAT from samples is usually ethyl acetate and the cleanup step may follow different techniques. Trucksess and Tang (1999) described a rapid method that fully abstains from the use of ethyl acetate as an extraction solvent and used a novel SPE approach. Arranz et al. (2005) conducted a collaborative trial for determination of PAT in apple juice and fruit puree. The extraction was based on ethyl acetate–hexane and used sodium bicarbonate and sulfate, following a SPE clean-up step. Ito et al. (2004) developed an analytical method that employs solid-phase extraction–liquid chromatography–mass spectrometry (SPE–LC–MS).

A sensitive method for quantification and confirmation of PAT in apple juice by gas chromatography/mass spectrometry (GC/MS) was developed by Tabata et al. (2004). PAT was extracted with ethyl acetate from a sample and then hexane was added to the concentrated extract solution. Significant amounts of insoluble impurities were filtered off, followed by further cleanup by SPE with combined silica gel and Florisil cartridges. The filtration step in a low polarity condition was very effective to remove the impurities in the sample extract solution. A syringe-cartridge SPE method was developed by Eisele and Gibson (2003) for determination of PAT in apple juice. A 2.5 ml portion of test sample was passed through a conditioned macroporous SPE cartridge and washed with 2 ml 1 percent sodium bicarbonate followed by 2 ml 1 percent acetic acid. A collaborative trial was conducted by MacDonald et al. (2000) to validate the effectiveness of a LC procedure for determination of PAT in both clear and cloudy apple juices and apple puree. The test portion of clear apple juice was directly extracted with ethyl acetate; cloudy apple juice and apple puree were treated with pectinase enzyme before extraction after back-extraction into sodium bicarbonate to remove interfering acidic compounds. A procedure combining diphasic dialysis extraction with *in situ* acylation and GC/MS determination was developed for detection and

quantification of PAT in apple juice (Sheu and Shyu, 1999). A simple and economical method has been developed for the determination of PAT in apple juice. The PAT is extracted with ethyl acetate in a diphasic dialysis system, and the extract is cleaned up by elution from a Sep-Pak cartridge (Prieta et al., 1993).

### 12.2.2. SEPARATION, DETECTION, DETERMINATION AND CONFIRMATION

Thin layer chromatography (TLC);  
High performance liquid chromatography (HPLC or LC); and  
Gas chromatography (GC).

A variety of methods of analysis for PAT in fruit and fruit juice has been developed: TLC (Prieta et al., 1992; AOAC, 1995; Vero et al., 1999), HPLC (Prieta et al., 1993; Rovira et al., 1993; Bartolomé et al., 1994; Gökmen and Acar, 1999; Trucksess and Tang, 1999; Eisele and Gibson, 2003; Gökmen et al., 2005; Iha and Sabino, 2005), GC (Tarter and Scott, 1991), GC/MS (Llovera et al., 1999; Sheu and Shyu, 1999; Roach et al., 2000; Rupp and Turnipseed, 2000; Tabata et al., 2004), LC/MS (Takino et al., 2003; Ito et al., 2004). Tsao and Zhou (2000) developed a method of capillary electrophoresis (CE) and Sewram et al. (2000) a method of LC/MS-MS. Although TLC methods predominated in the early seventies they later gave way to those based on HPLC. So, the most common method currently used to quantify PAT in fruit products is HPLC with ultraviolet detection. This is the official method adopted by AOAC Int. (1995) for apple juice (method 995.10) with a detection limit of 5 µg/l (Moake et al., 2005).

A method for the determination of PAT in apple juice was developed and validated in-house by Iha and Sabino (2005). The sample was extracted with ethyl-acetate-hexane and analyzed by LC equipped with a C<sub>18</sub> column and diode array detector. The mobile phase used for the quantification was water-ethanol, at a flow rate of 0.5 ml/min. The method showed a mean recovery of 84.8 percent the relative standard deviation obtained in the precision study was <7.7 percent. The quantification and detection limits were 7 and 3 µg/l. The ruggedness was evaluated by an intralaboratory experiment, in which five factors were studied, and only one was found to influence the observed results. The developed method is fast, practical, and simple; the solvents (except hexane) and reagents used were nontoxic. Trucksess and Tang (1999) developed a simple and rapid method. It takes 7 min to extract, using a SPE method, isolate, and purify the PAT from apple juice, unfiltered clear apple juice, and unfiltered cloudy apple juice. A portion of the test sample was passed through a macroporous copolymer cartridge and was washed with 1 percent sodium bicarbonate and then with 1 percent acetic acid. PAT was eluted with 2 percent acetonitrile in anhydrous ethyl and was determined by reversed-phase LC with UV detection at 276 nm. A rapid, simple, and economical method using a

limited amount of organic solvent is described for the determination of PAT in apple juice by Gökmen and Acar (1999). The extractor solvent is ethyl acetate and the extract is cleaned up by extraction with sodium carbonate solution. PAT is then determined by reversed-phase LC using a MicroPack C<sub>18</sub> columns and a variable-wavelength UV-Visible (UV-VIS) detector set at 276 nm. PAT and HMF were completely resolved by using water-acetonitrile (99:1 v/v) as the mobile phase. The detection limit is <5 µl.

Baritolomé et al. (1994) described a method for detection of PAT in apple juice and the simultaneous determinate PAT can be easily distinguished from compounds eluting under the same conditions. The detection limit for PAT is 8.86 µg/l.

The HPLC/UV procedure is routinely used for quantitative determination of PAT in apple products, but methods to confirm the presence of PAT usually include more specific detection techniques such as MS after LC or GC separations (Moake et al., 2005). The official PAT LC procedure was further examined (AOAC 995.10) by Roach et al. (2002). Juice or juice concentrate was extracted with ethyl acetate and cleaned up with sodium carbonate. PAT in the dried extract was determined by reversed-phase LC with UV detection (280 nm) in 1 percent tetrahydrofuran (THF) aqueous solution after evaporation of the ethyl acetate. An end-capped C<sub>18</sub> column was required to separate PAT from hydroxymethylfurfural. PAT was detected in approximately half of the >1000 extracts examined. Only ca 10 percent of the extracts contained PAT at levels greater than 50 µg/l. Some presumptive findings were confirmed by capillary GC/MS as the trimethyl silyl derivative using electron ionization or as underivatized PAT using negative ion chemical ionization. Trifluoropropylmethyl polysiloxane capillary columns provided superior GC of underivatized PAT compared to phenyl/methyl polysiloxane and methyl polysiloxane columns. A GC/MS method was developed by Rupp and Turnipseed (2000) for the confirmation of PAT and HMF extracted from apple juice. The extraction is based on the official AOAC method for liquid chromatographic analysis. Juice extracts are quickly and easily derivatized with bis (trimethylsilyl) trifluoroacetamide under mild conditions, and the trimethylsilyl ethers of the analytes are stable for at least several hours. The analytes are determined by GC/MS using an electron-impact source and selected ion monitoring of characteristic ion. The presence of PAT was confirmed at fortification levels of about 30–400 µg/l and naturally levels of about 80–400 µg/l. The presence of HMF was also confirmed at levels < or = 2 mg/l. An alternative approach based on the use of GC/MS developed by Llovera et al. (1999) is used to confirm the presence of PAT in apple juice. In the GC methods previously described, derivatization of PAT was always necessary in order to achieve good chromatographic detection. The use of electronic pressure control (EPC) and on-column injection avoids the need for PAT derivatization and allows a sensitive analysis of PAT. A detection limit of 4 µg/l in apple juice can be attributed to the method.

Sewram et al. (2000) developed an HPLC-MS-MS method with selective reaction monitoring (SRM) for the determination of PAT in apple juice samples to achieve a detection limit of 4 µg/l without derivation. A sensitive and selective method for quantification and confirmation of PAT in apple juice by

TABLE 12.1 Methods for patulin determination in apple juice.

Extraction	Cleanup	Technique	Confirmation	LOD ( $\mu\text{g/l}$ )	Reference
Ethyl acetate	Sodium carbonate	TLC	–	12 or 20	Vero et al. (1999)
Ethyl acetate	Silica gel	TLC	Rf	20	AOAC (2000)
Diphasic dialysis	–	TLC	–	50	Prieta et al. (1992)
Diphasic dialysis	SPE: silica gel	LC/UV	–	1	Prieta et al. (1993)
Ethyl acetate	SPE: silica gel	LC/UV	–	2	Rovira et al. (1993)
Ethyl acetate	Sodium carbonate	LC/UV	–	20	AOAC (2000)
Ethyl acetate	Sodium carbonate	LC/UV	–	4	Gökmen and Acar (1999)
SPE: Oasis HLB, Waters	Sodium bicarb. Acetic acid	LC/UV	GC/MS	5	Trucksess and Tang (1999)
1) SPE: Oasis HLB, Waters	–	LC/UV	–	3.6 <sup>b</sup>	Gökmen et al. (2005)
2) SPE: PVPP	2) SPE: C18	–	–	–	–
SPE: Oasis HLB, Waters	Sodium bicarb. Acetic acid	LC/UV	–	10	Eisele and Gibson (2003)
Ethyl acetate	Silica gel	GC-electron Capture	–	$\leq 10$	Tarter and Scott (1991)
Ethyl acetate	–	LC/UV	diode array	8.96	Bartolomé et al. (1994)
–	Dialysis derivatization	GC/MS	mass espect	10 <sup>b</sup>	Sheu and Shyu (1999)
Ethyl acetate	Sodium carbonate	GC/MS	mass espect	–	Roach et al. (2000) <sup>d</sup>
–	–	GC/MS	mass espect	4	Llovera et al. (1999)
Ethyl acetate	Silica gel	GC/MS derivatization	mass espect	–	Rupp and Turnipseed (2000) <sup>d</sup>

Ethyl acetate	Sodium carbonate SPE: GL-Pak and Aquisis	LC/MS	mass espect	4	Sewram et al. (2000)
Ethyl acetate HLB, Waters	PLS-3, GL Sciences	LC/MS	mass espect	–	Ito et al. (2004)
–	–	CE	diode array	3.8	Tsao and Zhou (2000)

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<sup>a</sup>detection limit

<sup>b</sup>quantification limit

SPE = Solid phase extraction

HLB = Hydrophilic lipophilic balanced

CE = Capillary electrophoresis

TLC = Thin layer chromatography

LC = Liquid chromatography

GC/MS = Gas chromatography/mass spectrometry

LC/MS = Liquid chromatography/mass spectrometry

LC/UV = Liquid chromatography/ultra-violet

GC/MS was developed by Tabata et al. (2004). By this method, PAT was precisely determined and confirmed down to the level of 1 and 5 µg/kg in samples, respectively.

The recent advances in technology of the technique and the established limits has provided an incentive for the development of faster and more specific analytical methods with lower detection limits. Sforza et al. (2006) have published a review of the use of MS in connection with chromatographic techniques for mycotoxin determination by considering separately the most diffuse class of mycotoxins. Although the selectivity of MS is unchallenged if compared to common GC and LC detection methods, accuracy, precision, and sensitivity may be extremely variable concerning the different matrices and instruments. The sensitivity issue may be a real problem in the case of LC/MS, where the response can be very different for the different ionization techniques.

There are actually two analytical method approaches needed, one for PAT regulation and another one for PAT research.

Table 12.1 summarizes the pertinent information about methods published in the literature.

### 12.3. CONCLUSIONS

A number of simple, sensitive, and reliable methods for the determination of PAT in fruit and fruit juice exist in the literature. The laboratory needs to establish a policy and procedure for the selection and use of analytical methods. The success of the analysis should not be dependent on the arbitrary selections of the method by the analyst (Garfield et al., 2000).

The most common method currently used to quantify PAT in fruit products is HPLC with UV detection or photodiode array detection. Actually, MS has been associated with both LC and CG, allowing for a conclusive confirmation of the toxin.

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# Detection and Determination of *Alternaria* Mycotoxins in Fruits and Vegetables

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## ABSTRACT

Species of *Alternaria* are known to produce a great number of secondary metabolites; however, only seven major toxins belonging to three different structural classes are known as possible food contaminants with a potential toxicological risk. These are alternariol (AOH), alternariol methyl ether (AME), altenuene (ALT), altertoxins I, II, and III (ALTX I, ALTX II and ALTX III), and tenuazonic acid (TA). Cleanup procedures of analytical methods include solid-phase extraction (SPE) for AOH, AME, ALT and ALTX I and solvent partition for TA. *Alternaria* mycotoxins have been determined by thin layer chromatography (TLC), gas chromatography (GC) and mainly by liquid chromatography (LC) with ultraviolet detection. Fluorescence and electrochemical detection have also been used. A Zn salt is usually added to the mobile phase. A technique that combines electrospray ionization (ESI) with negative ion detection and LC-mass spectrometry-mass spectrometry (LC-MS-MS) has overcome previous limitations of other analytical methods in terms of sensitivity and specificity and will provide a useful tool for the investigation of these toxins in fruit products. There are no international regulations for the *Alternaria* toxins in foods and whether the presence and co-occurrence of these metabolites constitute a problem in commercial products remains to be investigated.

## 13.1. INTRODUCTION

The genus *Alternaria* includes both plant pathogenic and saprophytic species that may damage crops in the field or cause post-harvest decay of fruits and vegetables. *Alternaria* spp. are the most common fungal species invading tomatoes (Harwig et al., 1979; Hasan et al., 1995) and are important pathogens

of various pome and stone fruits, citrus fruits, grapes, persimons, mangoes, melons, squashes, peppers, carrots, beans and other fruits and vegetables (Snowdon, 1990, 1992; Barkai-Golan, 2001). During their development in the host tissues, species of *Alternaria* are also known to produce a great number of secondary metabolites. Only seven major mycotoxins belonging to three different structural classes are known as possible food contaminants with a potential toxicological risk. These toxins are: alternariol (AOH), alternariol methyl ether (AME) and altenuene (ALT), which are dibenzopyrone derivatives; altertoxins I, II, and III (ALT X I; ALT X II, ALT X III), which are perylene derivatives; and tenuazonic acid (TA), a tetramic acid derivative. *Alternaria alternata*, the most common species in harvested fruits and vegetables (Barkai-Golan, 2001) as well as other *Alternaria* species are capable of producing a number of these mycotoxins in their hosts (Stinson et al., 1980, 1981; Özcelik et al., 1990; Bottalico and Logrieco, 1998; Tournas and Stack, 2001; Lau et al., 2003; Scott, 2004; Terminiello et al., 2006).

Other important metabolites produced by *Alternaria* species are phytotoxins, possessing phytotoxic properties and playing an important part in disease development in plants (Logrieco et al., 2003). Of these, the AAL phytotoxins which are produced by *A. alternata* f. sp. *lycopersici* are of special concern due to their structural and toxicological similarity to fumonisins, the carcinogenic mycotoxins produced by *Fusarium verticilloides* (Bottalico and Logrieco, 1992; Abbas and Riley, 1996; Abbas et al., 1996; Bottalico and Logrieco, 1998; Logrieco et al., 2003). Another phytotoxin, tentoxin, gained a particular interest since it can be isolated from *Alternaria* infected tomatoes and apples together with the characteristic *Alternaria* mycotoxins (Andersen and Frisvad, 2004).

Analytical methods for *Alternaria* mycotoxins detection in agricultural commodities and foods were last reviewed by Scott (2001). The present review intends to illustrate the current analytical techniques for the detection and determination of the main *Alternaria* mycotoxins in fruits and vegetables, which may pose serious effects on human and animal health following consumption (Scott, 2004). The mycotoxins were selected based on their relative importance and natural occurrence and grouped by similar chemical structure. These selected mycotoxins can also be used to illustrate the methodology applied in extraction, purification, separation and detection of related compounds.

## 13.2. TOXIN ANALYSIS

### 13.2.1. ALTERNARIOL, ALTERNARIOL METHYL ETHER AND ALTENUENE

AOH and AME are usually extracted from solid foods with organic solvents such as methanol, acetonitrile, ethyl acetate, chloroform, acidified chloroform-ethanol (4:1) and methanol-hexane (15:7) (Stinson et al., 1980,

1981; Logrieco et al., 1988; Özcelik et al., 1990; Scott, 2004). Cleanup may involve treatment with sodium bicarbonate, ammonium sulfate or lead acetate solutions and silica gel chromatography. SPE columns or cartridges are used for both extraction and cleanup in liquid foods like apple juice. C<sub>18</sub> and aminopropyl SPE columns in series are used for cleanup of apple juices and other fruits beverages (Lau et al., 2003; Scott, 2004).

Liquid chromatography has recently largely replaced GC and TLC for the determination of *Alternaria* toxins in food. After separation by reversed-phase LC, AOH and AME can be detected by UV, fluorescence, electrochemical detection or mass spectrometry (MS) (Scott, 2001).

Gas chromatography-mass spectrometry (GC-MS) is a reliable technique to confirm these mycotoxins as their trimethylsilyl and heptafluorobutyl derivatives (Scott et al., 1997), although high performance liquid chromatography (HPLC) with diode array detection (DAD) allows their confirmation without derivatization and purity assessment of the chromatographic peaks (Visconti et al., 1986, 1992; Delgado and Gómez-Cordovés, 1998).

A wavelength of about 256 nm at below pH 7 offers the greatest sensitivity in reversed-phase LC for AOH and AME. With this technique a detection limit of 1 ng/g in a method developed for the determination of AOH and AME in apple juice has been reached (Delgado et al., 1996; Scott, 2004).

A method for the determination of AME and AOH in tomato products has been described by da Motta and Soares (2000a). The method involves extraction with methanol, clarification with ammonium sulfate and partition to chloroform. Quantification was conducted by HPLC with DAD at limits of 2.0 ng/g for AME and 5.0 ng/g for AOH. The mobile phase was methanol-water (80:20) containing 300 mg ZnSO<sub>4</sub>·H<sub>2</sub>O. This method was used to investigate the occurrence of these toxins in tomato products in Brazil (da Motta and Soares, 2001) and in tomato puree in Argentina (Terminiello et al., 2006).

Fluorescence has also been explored for the determination of phenolic *Alternaria* toxins. Fluorescence is at least as sensitive as UV detection, but there can be interferences for AOH in apple juice extracts. A method for AOH in tomato paste had a detection limit of 9 ng/g, but overall recoveries were not good for AME (Scott, 2001).

AOH and AME were investigated in strawberries, blueberries, grapes and apples inoculated with toxigenic *Alternaria* strains. The toxins extraction was made with methanol and the detection by LC with a fluorescent detector. Excitation was set at 335 nm and emission at 440 nm. Elution with a solvent system of acetonitrile, water and acetic acid (50:50:1) was carried out. The detection limit in fruit was determined to be 0.01 µg/g (Tournas and Stack, 2001).

A binary gradient system of 0.02 percent of aqueous formic acid (pH 3) and methanol was used as mobile phase in HPLC analysis of AOH and AME in apple juice (Delgado and Gómez-Cordovés, 1998). For the HPLC-UV analysis of AOH, AME and ALT in apple tissues inoculated with toxigenic *Alternaria* a binary gradient of acetonitrile water with 50 µl/l of trifluoroacetic acid was employed. In this last study, the toxins were confirmed by HPLC-MS with electrospray ion source (ESI) and a flight mass spectrometer (Andersen et al., 2006).

These toxins have been determined in moldy tomatoes by HPLC-DAD, and a linear acetonitrile-water gradient with 50  $\mu\text{l/l}$  of trifluoroacetic acid has been employed as mobile phase (Andersen and Frisvad, 2004).

The extraction of naturally infected tomatoes and inoculated apples was made with ethyl acetate containing 1 percent of formic acid (Andersen and Frisvad, 2004; Andersen et al., 2006).

A method for the determination of AOH, AME and ALT X I in carrots and solid carrot-based products was developed (Solfrizzo et al., 2004; Solfrizzo et al., 2005). Carrots and carrot products were extracted with a mixture of acetonitrile-methanol water acidified to pH 3 with hydrochloric acid. The extracts were purified on a  $\text{C}_{18}$  column. Toxins were quantified by reversed phase HPLC with an UV-DAD detector set at 256 nm. The mobile phase consisted of two consecutive isocratic mixtures of acetonitrile-sodium dihydrogen phosphate solutions. Limits of detection were 0.01 and 0.005  $\mu\text{g/g}$ . Up to now this is the only method for *Alternaria* toxins in fruits and vegetables validated in an inter-laboratory study.

Liquid chromatography-mass spectrometry and LC-MS-MS confirmatory procedures based on electrospray (ESI) and atmospheric chemical ionization (APCI) with negative ion detection were applied to confirm the presence of AOH and AME in samples of grape juice, cranberry nectar, raspberry juice, red wine and prune nectar. Cleanup was made using the methodology for apple juice analysis developed by Delgado et al. (1996). ESI LC-MS-MS with negative ion detection and in multiple reaction monitoring mode offers higher sensitivity and specificity. Absolute detection was better than 4 pg per injection of both compounds (Lau et al., 2003). This methodology has been applied in a recent study for the determination of AOH and AME in wines, grape juices and cranberry juices. After cleanup on aminopropyl SPE columns, the toxins were first determined by reversed phase LC with UV detection. Positive extracts were re-analyzed by LC-MS-MS negative ion ESI in multiple reaction mode. Limits of detection were below 0.01 ng/ml (Scott et al., 2006).

### 13.2.2. ALTERTOXINS

Reversed phase LC of ALT X I can be carried out in the same mobile phase as AOH and AME. This toxin was investigated by LC-UV DAD in moldy tomatoes and apple tissues inoculated with toxigenic *Alternaria* with a binary gradient of acetonitrile water with 50  $\mu\text{l/l}$  of trifluoroacetic acid as mobile phase. In the apple tissues it was confirmed by HPLC-MS, ESI and a flight mass spectrometer. The extraction of naturally infected tomatoes and inoculated apples was made with ethyl acetate containing 1 percent of formic acid (Andersen and Frisvad, 2004; Andersen et al., 2006).

ALT X I in carrots and solid carrot-based products was investigated with the method described to analyze AOH and AME with a limit of detection of 0.02  $\mu\text{g/g}$  in these products (Solfrizzo et al., 2004; Solfrizzo et al., 2005). ALT X I and II are electroactive, and good sensitivity has been achieved by colorimetric and

amperometric techniques. Poor recoveries of ALT X I were obtained from silica gel C<sub>18</sub> SPE column, but this problem could be overcome by using polymer-based reversed phase SPE column (Scott, 2004).

### 13.2.3. TENUAZONIC ACID

For extraction of TA from food it is preferable to have an acidic aqueous organic extraction solvent (Scott, 2001). A commonly used cleanup for TA is extraction with bicarbonate solution followed by acidification with hydrochloric acid.

Tenuazonic acid could be determined by TLC (Schade and King 1984) and by GC (Scott et al., 1997). It could be determined in tomato paste at a level of 6 µg/kg by GC-MS single ion monitoring of its trimethylsilyl ether (Scott, 2004).

Since TA is not fluorescent, UV detection at 280 nm is necessary to determine it by reversed phase LC with UV detection (Scott and Kanhere, 1980) and UV-diode array detection (UV-DAD) as confirmation procedure. A metal ion chelating agent such as zinc sulfate should be added to the mobile phase to avoid peak tailing. Ion pair, anion exchange and ligand exchange LC have also been explored (Scott, 2004).

Tenuazonic acid was determined in tomato products together with AOH, AME and cyclopiazonic acid (da Motta and Soares, 2001). Methanol was used for extraction, the pH was lowered with hydrochloric acid and the extract was partitioned into chloroform. Sample extracts were analyzed by LC as described for AOH and AME determination. Chromatograms were recorded at 280 nm. The detection limit was 8.0 ng/g (da Motta and Soares, 2000b).

Tenuazonic acid was recorded together with AOH, AME, ALT, ATXI and tentoxin by HPLC-UV in apple tissues inoculated with toxigenic *Alternaria* strains and in naturally moldy tomatoes (Andersen and Frisvad, 2004; Andersen et al., 2006).

The method described by Solfrizzo et al. (2004) for AME and AOH was used for the analysis of TA but for the cleanup a polymeric OASIS hydrophilic lipophilic balanced (HLB) column was used and the detection was performed separately at 279 nm.

### 13.2.4. THE PHYTOTOXIN TENTOXIN

Tentoxin, which is a known phytotoxin produced by *Alternaria alternata* (Ramm et al., 1994), can be detected by TLC together with AOH, AME, ALT, ATX I and TA, the characteristic *Alternaria* mycotoxins. It was detected as a dark spot under UV light at 254 nm with a detection limit of 50 ng (Fabrega et al., 2002).

Tentoxin was extracted, determined and confirmed by HPLC-UV in apple tissues inoculated with toxigenic *Alternaria* strains and in naturally moldy tomatoes using the methods previously described for AOH, AME, ALT, ATX I and TA (Andersen and Frisvad, 2004; Andersen et al., 2006).

### 13.3. CONCLUSIONS

More work is clearly needed for the development of methods for determination and monitoring of *Alternaria* mycotoxins in fruits and vegetables in order to provide information on the level of human exposure to these foods.

No immunochemical methods have been developed for most of these mycotoxins and an immunoaffinity clean-up column would be of great value (Scott, 2004).

The technique that combined ESI with negative ion detection and LC-MS-MS has overcome previous limitations of other analytical methods in terms of sensitivity and specificity and will provide a useful tool for the investigation of these toxins in fruit products (Lau et al., 2003).

Many of the methods outlined in this review can be used or modified to meet specific analytical requirements but only one of these methods has been collaboratively evaluated.

There are no international regulations for the *Alternaria* toxins in foods and whether the presence and co-occurrence of these metabolites constitute a problem in commercial products remains to be investigated.

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# Chemical Control of Mycotoxigenic Fungi

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## ABSTRACT

To date, there is no information on direct chemical control of mycotoxins in fruit and vegetables. Thus, this chapter summarizes the state of the art of the use of chemical products for the prevention and control of mycotoxigenic fungi. Chemical control involves mainly the use of synthetic fungicides. While the control of diseases in fruits and horticultural commodities has become more difficult due to the appearance of resistant mutants of the target fungi, the use of fungicides has increased. The increasing concern of consumers and authorities regarding pesticide residues in foods is leading, however, to more strict control on the use of fungicides, to make it safer for the user, the environment, and the consumer. This has led to an increasing number of studies on the antifungal properties of extracts or compounds from different origins, including natural products, which have been carried out on mycotoxigenic species, including those involved in fruit and vegetable spoilage. The use of thiabendazole (TBZ) and imidazole fungicides, which is widespread and dates from some decades ago as a means for prevention of blue mold, prevents, although indirectly, patulin accumulation. Due to the appearance of resistant *Penicillium expansum* strains, research is focused at present on the search for new antifungals, either synthetic or not. Regarding ochratoxin A (OTA) prevention in grapes, there are no specific fungicides devoted to the inhibition of black aspergilli in vines; the existing trials report on the effect of pesticides usually applied to vineyards against black aspergilli infection and OTA accumulation. Finally, while many researches have been carried out in the past on the use of fungicides to prevent *Alternaria* rot in different fruits and vegetables, no attention has been paid to its mycotoxigenic ability.

## 14.1. INTRODUCTION

This chapter will cover the chemical control of *Penicillium expansum* (the producer of patulin in pome fruits), black aspergilli (the producers of ochratoxin A

in grapes and their derivatives), and *Alternaria* species (the producers of a variety of *Alternaria* mycotoxins) in various fruits and vegetables and their products, as well as attempts to minimize fungal contamination by means of alternative antifungals.

## 14.2. CHEMICAL CONTROL OF PATULIN PRODUCERS IN POME FRUITS

Blue mold rot is one of the most common diseases in cold-stored pome fruits. Since the widespread use of cold storage for fruit supply out of season, the search for chemical and biological control treatments has not ceased. Blue mold appears in wounded fruits. Wounds are caused during harvest and ulterior handling steps. Immersion dumping as a method of handling harvested apples and pears in the packinghouses enables accumulation of dirt and debris, and consequently spores of decay pathogens in the immersion dump water becomes a major problem. Chlorine or sodium orthophenylphenate is added to the dump water to control decay (Lennox et al., 2004). The most common facilities for postharvest fungicide treatment of fruit are drenchers (Fig. 14.1). During drenching, however, inoculum of *P. expansum* may be washed into fruit wounds sustained during harvest and transport to the packinghouse. Consequently, more wounds are exposed to pathogenic *P. expansum* inoculum in drenched than undrenched fruit (Lennox et al., 2004), if the microbial load of the circulating solution is not appropriately monitored and controlled.

Chemical control remains the principal way to reduce the incidence of blue mold in apples that are cold-stored for long periods. Intensive and exclusive use of benzimidazoles has caused selection of benzimidazole-resistant pathogens in most packinghouses. Since the discovery of thiabendazole (TBZ)-resistant *P. expansum* isolates in apple packinghouses, the search for alternative chemical control strategies have greatly increased. Fungicides are applied as prestorage fruit drenches or line sprays or, in some countries, in the field, several days before harvest. Many, but not all the synthetic fungicides are toxic substances and may cause serious damage to the ecosystem. The use of synthetic chemicals to control postharvest deterioration has been restricted due to their carcinogenicity, teratogenicity, high and acute residual toxicity, long degradation period, environmental pollution, and effects on food and other side effects on humans. It should be emphasized, however, that pesticide residues are not as toxic as mycotoxins (Kuiper-Goodman, 2004). Although the use of fungicides is somewhat discouraged, they are still used widely and biocontrol methods represent a very small share of the disease management market.

Presently, a few trials regarding chemical control have taken into account patulin as a variable; only fruit decay is considered in order to evaluate the effectiveness of chemical treatments. As a result, the current situation of the use and the research of antifungal products against *P. expansum* in pome fruits



FIGURE 14.1 Postharvest fungicide treatment in a drencher.

are summarized. However, while a higher percentage of decayed fruits generally imply a higher patulin contamination, the lesion sizes of decayed fruits do not necessarily correlate with the patulin content in those lesions.

### 14.2.1. SYNTHETIC FUNGICIDES

Systemic benzimidazole fungicides, benomyl, methyl 2-benzimidazole carbamate, and TBZ, were introduced into packinghouses in the 1970s to control postharvest decay caused by *P. expansum* and *Botrytis cinerea*. However, resistant strains to these fungicides developed soon. Development of resistance to commonly used fungicides within populations of postharvest pathogens has become a matter of much concern in recent years. Currently, imazalil is the most common used fungicide applied postharvest, as well as mixtures of imazalil plus folpet or other fungicide products such as iprodione, TBZ, etc. To harmonize the overall arrangements for authorization of plant protection products within the European Union, Directive 91/414/EEC concerning the

placing of plant protection products on the market came into force in 1993. This is achieved by harmonizing the process for considering the safety of active substances at a European Community level by establishing agreed criteria for considering the safety of those products. Product authorization remains the responsibility of individual Member States. The Directive provides for the establishment of a positive list of active substances, which have been shown to be without unacceptable risk to people or the environment. Active substances which are added to Annex I of the Directive as existing active substances are reviewed [under the European Commission (EC) Review Programme] and new ones authorized.

Imazalil is an imidazole-type systemic fungicide that inhibits the biosynthesis of ergosterol. It has a broad spectrum activity, wider than that of TBZ, and is active against resistant isolates to this type of products. A mixture of imazalil, folpet, and TBZ was applied in a drencher to apples that were subsequently wounded, inoculated with *P. expansum*, and cold-stored. The observation of lesion diameters after 6 weeks revealed a significant effect of fungicide treatment compared with controls. The efficiency of fungicide treatment depended, however, on the degree of apple ripeness, thus its effect could only be observed in the more ripen apples. Although lesions were evident, no patulin was detected at the end of cold storage (Morales et al., 2007a). Similar results were observed when apples were stored for 2.5 months under ultralow oxygen concentration conditions (Morales et al., 2007b). Unfortunately, the effect of fungicides on patulin accumulation could not be observed due to the short storage periods and the controlling effect of low temperatures; *P. expansum* is adapted to grow under low temperature levels, but patulin production seems to be highly inhibited. When those apples were transferred to room temperature before processing into apple juice, patulin accumulation was very high in only 3 days.

Other studies report the efficacy of fungicides (mainly newly developed ones) on *P. expansum* development on apples and pears, but unfortunately, patulin is not taken into account. Fludioxonil belongs to the chemical class of phenylpyrroles. This group of fungicides constitutes a new class of non-systemic fungicides. Fludioxonil was effective in preventing apple decay by TBZ-sensitive and TBZ-resistant isolates of *P. expansum*. Drench in fludioxonil solutions of 45–200  $\mu\text{g ml}^{-1}$  prevented decaying by inoculated *P. expansum* (Errampalli, 2004). The mode of action of this fungicide lies in the osmoregulatory signal transmission pathway of different pathogenic fungi. The fungicide increases the levels of glycerol in the mycelium fungi. The protein kinase, which may be involved in the regulation of glycerol biosynthesis, was shown to be inhibited by the phenylpyrroles (Rosslenbroich and Stuebler, 2000). Fludioxonil was very effective as co-treatment (protective) and as a post-inoculation (curative) treatment. At a concentration of 300  $\text{mg l}^{-1}$ , it gave complete control of postharvest blue mold for 105 days in controlled atmosphere (CA) storage, for 42 days in common cold storage, and for 6 days in shelf-life conditions. Fludioxinil at a concentration of 450  $\text{mg l}^{-1}$  gave 98 percent and 92 percent control of blue mold of apples in the simulated shelf-life studies after CA and common cold storage, respectively (Errampalli et al., 2005).

Scald is usually controlled by a postharvest drench treatment with the anti-oxidant diphenylamine (DPA). Apples treated with fludioxonil ( $3\text{--}5\text{ mg l}^{-1}$ ) together with DPA had higher disease incidence than apples treated with fludioxonil only. This might be attributable either to the surface effect of DPA carrying spores deeper into the wounds or to direct effects of DPA on apple physiology. The concentration of  $600\text{ mg l}^{-1}$  fludioxonil was required to control blue mold in the shelf-life study after cold storage in both DPA-treated and non-treated apples (Errampalli et al., 2006).

The protective effect of three fungicides, fludioxonil, cyprodinil, and a mixture of fludioxonil and cyprodinil, was evaluated against blue mold caused by TBZ-sensitive and TBZ-resistant isolates of *P. expansum*. The three fungicides were equally effective against TBZ-sensitive and TBZ-resistant isolates of *P. expansum*. No cross-resistance was observed between TBZ and fludioxonil or cyprodinil. Fludioxonil ( $45\text{ }\mu\text{g ml}^{-1}$ ), cyprodinil ( $50\text{ }\mu\text{g ml}^{-1}$ ), and fludioxonil + cyprodinil ( $50 + 75\text{ }\mu\text{g ml}^{-1}$ ) gave  $>97.0$  percent control for up to 1 month, but higher concentrations of fludioxonil and fludioxonil + cyprodinil were required to control blue mold for 62 days at  $4^{\circ}\text{C}$ . Fludioxonil and cyprodinil, which have modes of action different from each other and from that of the TBZ, have the potential to be effective components in the resistance management for postharvest control of apple blue mold (Errampalli and Crnko, 2004).

In a research work, the development of *Penicillium* rot in apple was significantly retarded by the  $\text{TiO}_2$  photocatalytic reaction (Maneerat and Hayata, 2006). When exposed to sunlight or ultraviolet light,  $\text{TiO}_2$  exhibits antimicrobial activity due to its strong oxidizing property.

A recent in vitro study showed that carbendazim, captan, and bupirimate inhibited patulin production by *P. expansum*, but to a smaller extent than its growth (Paterson, 2007).

In vivo testing of ammonium molybdate as a potential fungicide for the use in apples against the blue and gray molds at  $20^{\circ}\text{C}$  showed that ammonium molybdate ( $15\text{ mM}$ ) reduced lesion diameters of *P. expansum* by 84 percent. When apples treated with ammonium molybdate were stored at  $1^{\circ}\text{C}$  for 3 months, a significant reduction in severity and incidence of *P. expansum* was observed; the level of control was similar to, or greater than, that observed with the fungicide imazalil. When ammonium molybdate was applied as a preharvest treatment, a significant reduction in blue mold decay was observed after 3 months in cold storage (Nunes et al., 2001).

In a different approach, pyrimethanil was evaluated as a new postharvest fungicide both as a preharvest spray in field trials, compared with cyprodinil, and in postharvest dip trials, compared with TBZ (Sholberg et al., 2005). Preharvest treated apples were stored for 6 months at  $1^{\circ}\text{C}$ , wounded, inoculated, and incubated. Apples treated 20 days before harvest with pyrimethanil ( $80\text{ }\mu\text{g ml}^{-1}$ ) showed significantly smaller lesions than those treated with cyprodinil ( $230\text{ }\mu\text{g ml}^{-1}$ ). When applied 14 days before harvest, both pyrimethanil and cyprodinil were effective in controlling blue mold. Pyrimethanil was also very effective postharvest in controlling decay in wounds inoculated

with *P. expansum* conidia at rates as low as  $250 \mu\text{g ml}^{-1}$ . No deleterious effects on fruit quality were reported. The mode of action of this fungicide is different from other fungicides that do not belong to the anilinopyrimidine group of fungicides and likely involves shutting down the cell's ability to manufacture methionine (Masner et al., 1994). This fungicide constitutes a good alternative for the control of both blue and gray mold, applied both pre- and postharvest.

### 14.2.2. ORGANIC FRUITS AND PATULIN CONTENT

There is some evidence that the reduced use of fungicides and pesticides may lead to a greater contamination by toxins in organic than in conventional food, although some studies have found the opposite trend. Experiments by Malmauret et al. (2002) provided evidence for increased apple contamination in organically produced fresh apples. There are reports on studies carried out in Europe (France, Italy) mentioning that organic apple juices contain more patulin than the conventional products (Beretta et al., 2000). In a recent study carried out in Belgium, attention was paid to the influence of several possible confusion factors, such as industrial versus handicraft juices, clarified versus turbid juices, local versus imported juices, and, to a lesser extent, organic versus conventional juices. However, looking at the various factors capable of affecting contamination, no significant differences could be found even if it appeared that the mean content in industrial juices was lower than that found in juices of handicraft origin (Tangni et al., 2003). In contrast, in another study carried out in Belgium on 22 organic and 36 conventional apple juices, the organic juices contained significantly higher amounts of patulin (means of  $33.4 \mu\text{g l}^{-1}$  in organic versus  $8.1 \mu\text{g l}^{-1}$  in conventional) (Baert et al., 2003).

### 14.2.3. ALTERNATIVE ANTIFUNGALS

Fungicides are the primary means of controlling postharvest diseases in fruits and vegetables, and it is generally accepted that production and marketing of these perishable products would not be possible without their use (Ragsdale and Sisler, 1994). However, as harvested fruits and vegetables are commonly treated with fungicides to retard postharvest diseases, there is a greater likelihood of direct human exposure to them than to chemicals that are applied solely to protect foliage (Tripathi and Dubey, 2004). The side effects of synthetic fungicides means that alternative strategies need to be developed for reducing losses due to postharvest decay, which are perceived as safe by the public, and pose negligible risk to human health and environment (Wilson et al., 1999). The use of non-chemical methods and non-selective fungicide treatments may provide a part of this need.

The effectiveness of 5 percent and 10 percent hydrogen peroxide in growth inhibition of *P. expansum* in agar diffusion assay was total, and its minimum

inhibitory concentration (MIC), as determined by the agar and broth dilution assays, was less than 0.025 percent. These results indicate that the application of small quantities of hydrogen peroxide to the apple skin might be an alternative to fungicides in the elimination of *P. expansum* (Venturini et al., 2002).

Replacement of synthetic fungicides by natural products (particularly of plant origin), which are non-toxic and specific in their action, is gaining considerable attention (Table 14.1). A review on the exploitation of natural products such as flavor compounds (e.g., acetaldehyde, benzaldehyde, hexanal, etc.), acetic acid, jasmonates, glucosinolates, propolis, fusapyrone and deoxyfusapyrone, chitosan, essential oils, active principles of some plants, and plant extracts in the management of decay of fruit caused by plant pathogenic fungi was published by Tripathi and Dubey (2004).

Pears exposed to trans-2-hexenal 24–72 h after inoculation with *P. expansum* showed significant reduction in occurrence compared with control. This suggested that germinating conidia of *P. expansum* are probably more susceptible than non-germinating conidia. The exposure was effective among pears stored at –1°C for 60 days after treatment. A 2 h exposure time was enough to reduce infection significantly (84 percent), but 8 h were required for patulin reduction (78 percent). Color, firmness, soluble solid content, and titratable acidity were not significantly affected by trans-2-hexenal treatment, although some off-odors and off-flavors were perceived (Neri et al., 2006). 10-oxo-trans-8-decenoic acid and 1-octen-3-ol were in vitro inhibitory to *P. expansum* growth. An additive effect was observed when both compounds were added at a combined concentration of ≥1.25 mM; when both were added at a combined concentration

TABLE 14.1 Alternative antifungals with proven activity against mycotoxigenic fungi.

Products	Target fungus	Substrate	Reference
Flavor compounds			
Trans-2-hexenal	<i>Penicillium</i>	Pears	Neri et al. (2006)
1-octen-3-ol	<i>expansum</i>	Synthetic medium	Okull et al. (2003)
Isothiocyanates	<i>P. expansum</i> <i>Alternaria</i> <i>alternata</i>	Peppers, tomatoes	Troncoso-Rojas et al. (2005a,b)
Organic acids			
Acetic acid	<i>P. expansum</i>	Apples, pears	Sholberg (1998)
Formic acid	<i>P. expansum</i>	Apples, pears	
Propionic acid	<i>P. expansum</i>	Apples, pears	
Essential oils			
Thyme	<i>P. expansum</i>	Synthetic medium	Venturini et al. (2002)
Cassia	<i>A. alternata</i>	Synthetic medium	Feng and Zheng (2007)
Citral	<i>P. expansum</i>	Synthetic medium	Venturini et al. (2002)
Neem extract	<i>P. expansum</i>	Synthetic medium	Mossini et al. (2004)
Citrus sinensis	<i>P. expansum</i>	Synthetic medium	Sharma and Tripathi (2006)
Chitosan	<i>P. expansum</i>	Apples	Capdeville et al. (2002)
	<i>A. alternata</i>	Tomatoes	Bashkiria Reddy et al. (1998, 2000)



of 2.5 mM, mycelial growth was inhibited by 48.8 percent and 72.8 percent at pH 5.6 and pH 3.5, respectively (Okull et al., 2003).

Acetic, formic, and propionic acids, when used in vapor form ( $1.2\text{--}2.5\ \mu\text{l l}^{-1}$ ), are all very effective in destroying surface-borne fungal spores that cause decay of fruits. In particular, when *P. expansum*-inoculated apples and pears were fumigated, injured, and placed at 20°C, decay caused by *P. expansum* was reduced from 98 percent to 16 percent, 4 percent, or 8 percent by acetic, formic, and propionic acids, respectively, without injury to the fruit (Sholberg, 1998).

Evaluation of the essential oils (thymol, eugenol, citral, and cineole) and vanillin, in vitro against *P. expansum*, indicated that thymol and citral showed the greatest inhibitory effects (Venturini et al., 2002). In vitro experiments on *P. expansum* growth and patulin production in a liquid medium containing neem extracts showed no inhibition of fungal growth; on the other hand, treating cultures with increasing levels of neem extracts resulted in detection of decreasing levels of patulin. At the level of  $50\ \text{mg ml}^{-1}$  neem extract the toxin was reduced by 96 percent. The extract's antifungal activity may be associated with highly bioactive compounds that may change the fungus' secondary metabolism (Mossini et al., 2004). Growth of *P. expansum* in vitro has been completely inhibited by *Citrus sinensis* oil ( $>500\ \text{ppm}$ ) applied by the poisoned food technique; volatile activity of this oil also showed important inhibition of growth (Sharma and Tripathi, 2006).

Chitosan, a given name to a deacetylated form of chitin, was proved to control numerous pre- and postharvest diseases on various horticultural commodities. In addition to its direct microbial activity, studies suggest that this compound induces a series of defence reactions correlated with enzymatic activities of glucanohydrolases, phenolic compounds, and synthesis of specific phytoalexins with antifungal activity. In summary, chitosan is reported to restrict, to some extent, fungal penetration and induce the formation of different structural barriers (Bautista-Baños et al., 2006). Chitosan has been used as an alternative control agent against blue mold in harvested 'Red Delicious' apple fruit and has been shown to induce resistance in the fruit rather than merely inhibiting the pathogen directly (Capdeville et al., 2002).

Phytocompounds are expected to be far more advantageous than chemicals for sheer magnitude of complexity, diversity, and novelty of chemicals and their reactions. As they are biodegradable in nature, non-pollutant, and possess no residual or phytotoxic properties, these natural products have the potential to be safe fungicides to replace the synthetic ones.

The encouraging results of the use of natural products to control postharvest fungal rotting indicate that we should be able to develop natural fungicides that would be as effective as synthetic fungicides, and presumably safer for man and the environment. First, these products should be properly tested for their practical potency based on in vivo trials, organoleptic tests, and safety limit profile (Tripathi and Dubey, 2004).

Alternatives to fungicides in fruit disease control have arisen with the discovery of inducible resistance in fruit against infection by pathogens, and

the induction of fruit resistance was evaluated as a promising approach for the control of postharvest diseases. The disease incidence and lesion area in pears inoculated with *P. expansum* significantly decreased by the infiltration with 0.5 mM acibenzolar-S-methyl (ASM) after harvest, and the duration of protection conferred lasted over 15 days. ASM did not directly inhibit the mycelial growth of *P. expansum* in vitro. However, ASM treatment significantly enhanced activities of the main defence enzymes including peroxidase, phenylalanine ammonia-lyase, and chitinase (Cao et al., 2005).

### 14.3. CHEMICAL CONTROL OF OCHRATOXIN A PRODUCERS IN GRAPES

#### 14.3.1. SYNTHETIC FUNGICIDES

With regard to mycotoxins, until 1996 grape was considered as a safe product, virtually free of fungal toxins. Zimmerli and Dick (1996) detected OTA during a survey carried out on wine samples of different origins in Switzerland. The causal agents of this contamination were species of *Aspergillus* section *Nigri*, and in particular *Aspergillus carbonarius* and *Aspergillus niger*, growing on grapes in the field. The so-called *black rot of grapes* is a disease particularly severe in wine-producing countries of South Europe, like France, Italy, Greece, and Spain, as well as in North African countries as Egypt, Algeria, Tunisia, and Morocco. The fungus survives on plant debris in the soil, and the conidia are disseminated in vineyards by warm (25–30°C) air currents (Logrieco et al., 2003). Because of its black spores, this fungus is highly resistant to sunlight and survives sun-drying, leading to OTA contamination in raisins too.

Several in vitro or field trials have been carried out on the influence of fungicides on OTA-producing fungi and subsequently on OTA accumulation in grapes and/or wine, but at the moment no specific application of fungicides is systematically applied to control black aspergilli in these products.

Infection is initiated mainly through accidental injuries, more frequent in mature berries, and then under warm conditions it spreads throughout the bunch. The injuries could also be produced by insects (mainly by the grape berry moth, *Lobesia botrana*, or other insects as *Eudemis* or *Cochylis* spp.), excessive irrigation, rain damage, or careless handling. In fact, the influence of *L. botrana* on OTA contamination of grapes has been demonstrated by different assays. Merrien (2003) observed the successful effects of the use of insecticides like Lufox (carbamate-type insecticide containing lufenuron and fenoxycarb), Decis (a pyrethroid insecticide containing deltamethrin), and Bt (*Bacillus thuringiensis*) for lowering OTA content of wines. Similarly, Kappes et al. (2005) showed that treatment of grapes with products with ovicidal activity as lufenuron, alone or with fenoxycarb, leads to a strong reduction of OTA (up to 89 percent reduction) in musts. Cozzi et al. (2006) confirmed the interaction

between *L. botrana*-damaged berries and OTA contamination in the field. *Aspergillus*-rotted berries with *L. botrana* larvae damage had higher and more uniform populations of black aspergilli when compared with *Aspergillus*-rotten samples (grape without *L. botrana* larval injuries), and the same trend was also observed for the OTA contamination levels in grapes.

Several in vitro trials have been developed to test the efficacy of different fungicides on black aspergilli development and OTA production. Belli et al. (2006) screened 26 fungicides, at doses recommended by the manufacturers on a grape-like synthetic medium at 20 and 30°C, finding that 13 of them completely inhibited *A. carbonarius* growth at both temperatures and nine significantly reduced growth rate (Table 14.2). Fungicides that prevented fungal growth obviously prevented OTA production, but not all the fungicides that reduced growth reduced OTA synthesis. In general, fungicides that contained copper or strobilurins reduced both growth and OTA production, contrary to sulfur fungicides. The best results on in vitro *A. carbonarius* control were achieved with Switch (cyprodinil 37.5 percent plus fludioxonil 25 percent) and Chorus (cyprodinil 50 percent), as they showed the minimum effective threshold concentrations (Belli et al., 2006). Natamycin, a fungicide produced by *Streptomyces natalensis*, was shown to control *A. carbonarius* growth and OTA production in in vitro studies on a red grape extract medium, in a way that depends on water activity and temperature (Medina et al., 2005); authors postulate that a dose of 10 ppb is probably enough to control fungal development under a wide variety of conditions.

In vitro trials with *A. carbonarius*-inoculated table grapes (Red Globe variety) showed that both infection and OTA production were reduced when using Switch and Quadris (azoxystrobin 25 percent), whereas treatment with Topas (penconazole 10 percent) also showed a clear reduction in OTA production (Belli et al., 2006).

TABLE 14.2 Fungicides with demonstrated in vitro development inhibition of *Aspergillus carbonarius* (adapted from Belli et al., 2006).

Fungicide	Company	Composition	Dose
TOPAS	Syngenta	Penconazole 10% p/v	0.35 ml l <sup>-1</sup>
Experimental product	Syngenta	CGA302130	2 ml l <sup>-1</sup>
GEOXE	Syngenta	Fludioxonil 50%	0.5 g l <sup>-1</sup>
SWITCH	Syngenta	Cyprodinil 37.5% plus fludioxonil 25%	1.8 g l <sup>-1</sup>
CHORUS 50 WG	Syngenta	Cyprodinil 50%	2 g l <sup>-1</sup>
RIDOMIL GOLD COMBI	Syngenta	Folpet 40% plus mefenoxam 5% WP	2 g l <sup>-1</sup>
EUPAREN M	Bayer	Tolyfluanid 50% p/p	1.75 g l <sup>-1</sup>
FOLICUR 25EW	Bayer	Tebuconazole 25% p/v	0.70 ml l <sup>-1</sup>
CAPLUQ-50	Luqsa	Captan 50% p/p	3.5 g l <sup>-1</sup>
CARBENLUQ-50	Luqsa	Carbendazim 50% p/p	0.6 g l <sup>-1</sup>
MANCOZEB 80	Luqsa	Mancozeb 80% p/p	3 g l <sup>-1</sup>
TMTD 80	Luqsa	Tiram 80% p/p	2.5 g l <sup>-1</sup>
ZICOLUQ 320	Luqsa	Copper oxychloride 22% p/p plus mancozeb 17.5% p/p	5 g l <sup>-1</sup>

Earlier studies showed that combinations of Euparen (a sulfamide-type fungicide) and Mycodifol (Karadimcheva, 1978), or captan (Tandon et al., 1975) were effective against black aspergilli on grapes. Molot and Solanet (2003) found that Switch, Scala (containing pyrimethanil), and Mikal (containing fosetyl-Al and the dicarboximide folpet) were effective in lowering fungal colonization and OTA content of wines.

Different treatments have demonstrated their ability to control black rot of grapes and OTA production. It was frequently found that data obtained about the control of black aspergilli by fungicides have been a secondary result of chemical treatments specifically designed to control other fungi more frequently found in grapes. Switch, originally designed to control *Botrytis* and *Sclerotinia* infection in different crops, is perhaps the only treatment directly registered against *Aspergillus* on grapes. This chemical is a preformulated mixture of cyprodinil (37.5 percent) and fludioxonil (25 percent), being the first anilinopyrimidine fungicide with some systemic properties, which is taken up into the cuticle and waxy layers of leaves and fruits. On the other hand, fludioxonil is a phenylpyrrole fungicide which stays on the leaf and fruit surfaces to provide contact activity.

In a field study developed in the year 2000 on vineyards of three Italian regions, treatment of grapes with azoxystrobin reduced OTA concentration of wine by 96.5 percent, and a 88 percent reduction was observed when grapes were treated with the organic pesticides dinocap plus penconazole (Lo Curto et al., 2004).

Prêtet-Lataste et al. (2005) found that at least three fungicides have a high efficiency to reduce the OTA contamination in musts: Switch, mepanipyrim, and pyrimethanil. Using these fungicides at veraison or 15 days later, depending on the year, may reduce *Aspergillus* contamination by 80 percent. Kappes et al. (2005) found similar results obtaining maximum reductions in OTA concentration in musts combining a botrycide treatment such as Switch with an insecticide treatment such as lufenuron plus fenoxycarb.

Dimakopoulou et al. (2005) found that Switch 0.1 percent was able to reduce sour rot caused by black aspergilli in the grapevine variety Agiorgitico of Korinth region, whereas Cupravit 0.5 percent showed worse results. Similar results were obtained by the same group using the grape varieties Cabernet Sauvignon and Grenache Rouge in Rhodes island, showing that two Switch applications (first treatment at veraison and second one 20 days before harvesting) significantly reduce *Aspergillus* populations and OTA level in musts (Antonioni et al., 2005).

Trials developed on four Spanish vineyards during 2004 evaluated the effect of application of Switch and Chorus on the mycoflora of grapes, especially on ochratoxigenic fungi (Bellí et al., 2007). Grape varieties studied were Tempranillo, Malvasía, Airén, and Macabeo. Different doses and application times were evaluated, always combined with doses of Lufox, an insecticide for the control of *L. botrana*, and with basic cover treatments to reduce the apparition of other pests to *Aspergillus* or *Botrytis*. Switch applied at veraison and 21 days before harvest was the most effective treatment to prevent black aspergilli in grapes.

The exact moment of application of Switch is determinant in the efficacy of treatment. It seems that Switch is effective in reducing *Aspergillus* rots when

applied shortly before veraison, with follow-up sprays before harvest conferring additional reductions. In Australia, regulations prohibit the application of Switch within 60 days of harvest, and typically this chemical is applied at flowering or before bunch closure. Preliminary trials made with Semillon vines have shown that this early application before bunch closure had little effect on *Aspergillus* rots in bunches artificially inoculated with *A. carbonarius* (Leong et al., 2006).

With regard to raisins, Tjamos et al. (2004) demonstrated that Switch was able to control the incidence of sour rot caused by *Aspergillus* spp. in Corinth raisins and wine-producing cultivars (Grenache Rouge and Cabernet Sauvignon) in two consecutive growing seasons (2002–2003) and geographical areas of Greece. Late Switch applications were more efficient compared with the early ones. As parallel treatments with Chorus were ineffective, authors concluded that fludioxonil is responsible for the effectiveness of Switch. In addition, carbendazim proved to be extremely ineffective in controlling sour rot of grapes.

Although the use of pesticides seems one of the simplest and most effective methods to control different microorganisms of vine, there is also an increasing concern regarding the impact of fungicides on human health, on the appearance of resistances, and on the environment. However, it is possible to predict the residual levels of pesticides on a bunch of grapes and limit them to safe levels, and most of the times the residual levels in vinification are below the detection limit. On the other hand, biological control is an alternative option that is being explored intensely. Combination of preventive measures for both infection and mycotoxin production is the challenge for the future of fungal control of grapes.

#### 14.4. CHEMICAL CONTROL OF *ALTERNARIA* IN FRUITS AND VEGETABLES

*Alternaria* species are common pathogens of fruits and vegetables causing important losses in pre- and postharvest processing. Species of this genus, like *Alternaria alternata* and *Alternaria radicina*, can produce mycotoxins and phytotoxins. However, *A. alternata* is the main mycotoxigenic species of this genus because it occurs at higher numbers of food crops and is capable of producing the mycotoxins tenuazonic acid, alternariol, alternariol methyl ether, and alternotoxin. Moreover, *A. alternata* f. sp. *lycopersici* can produce the AAL phytotoxins (Solfrizzo et al., 2005).

Production of *Alternaria* mycotoxins in whole tomatoes, apples, oranges, and lemons infected with *Alternaria* indigenous to these fruits was demonstrated for the first time by Stinson et al. (1981). Tomato seems to be the crop most likely to be contaminated by important concentrations of *Alternaria* mycotoxins (Stinson et al., 1981). Carrots, which are very susceptible to *Alternaria* spoilage, do not represent a hazard for consumers as far as mycotoxins are concerned (Solfrizzo et al., 2005).

Reports about the chemical control of toxigenic species of *Alternaria* and their mycotoxins are very scarce. The effects of some chemical products for controlling the *Alternaria* brown spot of citrus cultivars and other fruits

and crops have been reported (Biggs et al., 1993; Solel et al., 1997; Prusky et al., 2002; Reuveni and Sheglov, 2002; Troncoso-Rojas et al., 2005a,b; Prusky et al., 2006, Reuveni, 2006). Control of *Alternaria* damages in food crops should also control the mycotoxin production.

#### 14.4.1. SYNTHETIC FUNGICIDES

Chemical control against *Alternaria* is usually carried out in the field. Several families of fungicides, such as dicarboximides, carbamates, benzimidazoles, and triazoles, are used as seed or foliar treatments in the protection of cruciferous crops against pathogenic *Alternaria* spp. in many countries. Triazoles (difenoconazole and tebuconazol), strobilurins (pyraclostrobin and azoxystrobin), and chlorotalonil are fungicides recommended to control *Alternaria* in tomatoes. The dicarboximide iprodione was the most effective fungicide to control *Alternaria* on citrus, where copper and mancozeb are very effective as well. Approaches that are based on using iprodione, as a mixture or in alternation with other fungicides, have been proposed by Solel et al. (1997) to control *Alternaria* brown spot of *Minneola tangelo*.

Azoxystrobin, pyraclostrobin, trifloxystrobin, difenoconazole, bromoconazole, and polyoxin B reduced the in vitro spore germination and growth of *A. alternata*. These fungicides are effective in apple orchards to control the moldy-core development produced by *Alternaria* in Red Delicious apple fruit (Reuveni and Sheglov, 2002; Reuveni, 2006). In addition, the imidazole prochloraz was found to be effective in vitro on mycelial growth inhibition of different species of *Alternaria*, including *A. alternata* (Iacomi-Vasilescu et al., 2004).

Different postharvest treatments to control *Alternaria* rot are recommended in fruits. The combination of prochloraz in the hot water brushing further reduced the incidence of decay of mango fruit. According to the authors (Prusky et al., 1999), this leads to eradication of latent infections initiated in the orchard and consequently prevents fungal colonization. This decay depends on the level of quiescent infection of *A. alternata* in the fruits at harvest (Prusky et al., 2002). The effectiveness of prochloraz treatment in controlling quiescent infections of *A. alternata* in stored mango and persimmon fruit was improved after combination with hydrochloric acid (HCl) (Prusky et al., 2006). A prestorage chlorine dip treatment prevented the postharvest development of *A. alternata* in persimmons during storage (Prusky et al., 2001).

#### 14.4.2. RESISTANCE

In general, a partial loss of efficacy of some spray programs has been observed. Decline in the control of the disease might be related to several factors, including emergence of fungicide resistance.

Isolates of *A. alternata* collected from a field site which had repeatedly been treated with iprodione exhibited a high level of resistance to iprodione and to

the phenylpyrrole fungicide fludioxonil, in plate assays and in the field (Solel et al., 1996; Dry et al., 2004). Comparison of nucleic acid sequences from the fungicide-sensitive and fungicide-resistant *A. alternata* isolates confirmed the presence of mutations leading to premature termination of the translated histidine kinase protein (Dry et al., 2004).

### 14.4.3. ALTERNATIVE FUNGICIDES – NATURAL COMPOUNDS

Other alternatives include natural compounds with antimicrobial properties. The natural compounds tested to control *Alternaria* are chitosan, some essential oils, and isothiocyanates (Table 14.1).

Chitosan has been assayed in in vitro experiments and in tomato fruits in order to assess the effects on growth, toxin production, and various pathogenic factors of *A. alternata* (Bashkiria Reddy et al., 1998, 2000). Chitosan retarded the apparition of visible black old lesions and inhibited production of oxalic and fumaric acids (chelating agents) and the synthesis of mycotoxins such as alternariol and alternariol monomethylether by the fungus. This compound also induced the production of the phytoalexin rishitin in tomato tissue.

Another alternative could be the use of essential oils. Most of the essential oils have been reported to inhibit fungi in vitro (Bellerbeck et al., 2001; Velluti et al., 2004). According to the findings of Feng and Zheng (2007), the cassia and thyme oils exhibited antifungal activity against *A. alternata*. Cassia oil inhibited spore germination and germ tube elongation, and at 500 ppm reduced the percentage of decayed tomatoes. Because of its effect on tomato flavor, more work on quality parameters of tomatoes after storage is required.

Isothiocyanates, the antifungal properties of which have been reported since 1937 (Walker et al., 1937), have been tested against *Alternaria* by Troncoso-Rojas et al. (2005a,b). These compounds inhibited completely *Alternaria* growth in vitro, and when applied to control fungal rot on packed bell pepper and packed tomatoes, they showed better results than the synthetic fungicide tested, with no adverse effects on fruit quality.

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# Physical Control of Mycotoxigenic Fungi

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## ABSTRACT

Heat treatments such as hot-water dips, vapor heat, dry heat or curing, and hot-water rinsing and brushing (HWRB) alone, or in combination with modified or controlled atmosphere, biocontrol agents, ethanol, bicarbonate salts, and even low fungicide dosages, have been found to control decay fungal development after harvest without residues.

This chapter will summarize the information published during the last decade on heat treatments as direct sole treatments or in combination with the other means mentioned above to control decay development and mycotoxins accumulation in agricultural products in vitro and in vivo.

## 15.1. INTRODUCTION

A basic approach to control mycotoxin contamination in fruits and vegetables intended for consumption is the suppression of postharvest fungal pathogens responsible for its production. The present chapter describes the possibilities of using physical means and particularly heat treatments, to suppress the development of *Aspergillus*, *Penicillium*, and *Alternaria* species responsible for the accumulation of mycotoxins.

*Aspergillus* molds are found worldwide. The genus includes over 150 species, some of which may infect fruits and vegetables and are capable of producing mycotoxins that adversely affect humans and animals health via consumption. These include aflatoxins and ochratoxin A, both of which have been found to be carcinogenic. The frequency and relative importance of these infections are on the rise in all developed countries (Stevens et al., 2000).

*Penicillium expansum*, the causal agent of the blue mold rot, is a common postharvest pathogen on apples and pears worldwide. This disease is an economic concern not only to the fresh-fruit industry but also to the

fruit-processing industry because some strains of *P. expansum* produce the mycotoxin patulin, which can rise to unacceptable levels (Wilson and Nuovo, 1973).

*Alternaria* is a cosmopolitan fungus commonly isolated from plants, soil, food, and outdoor and indoor air environment. The genus *Alternaria* currently contains more than 50 species, among which, *A. alternata* is the most common one. *Alternaria* species are some of the most prodigious producers of toxic secondary metabolites, producing over 70 compounds of varying toxicity. Some of these metabolites are powerful mycotoxins with mutagenic and teratogenic properties, and have been linked to certain forms of cancer (Andersen et al., 2001).

The problem of fresh produce and processed products with mycotoxins is of current concern and has received a great deal of attention during the last four decades. The frequent incidence of these toxins in fresh or processed agricultural commodities has a potential negative impact on the economies of the affected regions, especially in the developing countries where harvest and postharvest technologies are not adequate enough to control mold growth and development (Rustom, 1997).

Weather conditions prior to harvest, type and amount of fungicide used, ecology of the key fungal species, and moisture content of crops at harvest are all key aspects which need to be considered in determining the level of fungal and toxin contamination. Postharvest storage, transport, and processing are all important stages in the food chain which need to be monitored. This type of information is important for the development of Decision Support Systems (DSS) for predicting the level of risk in a particular production season from mycotoxin contamination which may exceed the legislative limits (Magan, 2006).

Preharvest contamination can be reduced by introduction of good agricultural practices that limit the growth of mycotoxigenic fungi (Tamm, 2001). Postharvest contamination can be minimized by application of proper postharvest practices such as curing, sorting, drying, and heat treatments. However, mycotoxins have high decomposition temperatures and therefore, are very stable to heat inactivation (Betina, 1989). Hence, destroying the spores and mycelia of the mycotoxigenic fungi by heat treatments, in combination with other means, might reduce or eliminate fungal proliferation and reproduction of the toxin.

Prestorage heat treatments to control decay development during storage and marketing period are often applied for a relatively short time (minutes), because the target decay-causing agents are found on the surface or in the first few cell layers under the skin of the fruit or vegetable (Barkai-Golan, 2001). Heat treatments against pathogens may be applied to the fresh harvested produce in several ways: by hot-water dips, by vapor heat, by hot dry air (Lurie, 1998) and by a short hot-water rinsing and brushing (HWRB) (Fallik, 2004). However, hot water is an effective heat transfer medium and, when properly circulated through the load of fruit, establishes a uniform temperature profile more quickly than either vapor or dry heat (Lurie, 1998).

The chapter will focus on three major mycotoxin production fungal genera: *Aspergillus*, *Penicillium*, and *Alternaria* and the effect of heat treatments, alone, or in combination with other means, on the growth and development of those fungi, in vitro and in vivo, during the last decade.

## 15.2. IN VITRO

There is considerable variation in the sensitivity to high temperature among various fungi (Barkai-Golan and Phillips, 1991; Fallik et al., 1996; Karabulut et al., 2002). However, pathogen kill is not always proportional to the temperature-time product of the treatment, although reports have indicated a linear relationship between the logarithm of the decimal reduction time and the temperature of the heat treatment. Vegetative cells and conidia of most fungi are inactivated when exposed to a temperature of 60°C for 5–10 min in vitro (Civello et al., 1997). Spore germination and germ tube elongation were found to be more sensitive to heat treatments than dormant spores (D'hallewin et al., 1997). Hot-water treatments were thus found to be ineffective in killing dormant spores (D'hallewin et al., 1997).

Studying the effectiveness of hot-water dipping on the control of *A. alternata* on sweet red pepper quality, spore germination and germ tube elongation in vitro were found inversely related to the duration of exposure or to the range of temperature used. The  $ET_{50}$  for spore germination for *A. alternata* was found to be 8.8, 4.2, and 1.4 min, at 45, 50, and 55°C, respectively, while the  $ET_{50}$  for germ tube elongation for *Alternaria* spores was 7.2, 2.5, and 1.6 min, at 45, 50, and 55°C (Fallik et al., 1996). Mycelial growth and percent spore germination of *P. expansum*, in vitro, were inversely proportional to the length of time of exposure to various temperatures. The  $ET_{50}$  for spore germination was 42, 34, and 20 h at 38, 42, and 46°C, respectively, while the  $ET_{50}$  for mycelial growth was 48, 44, and 36 h at those temperatures (Fallik et al., 1995). Exposing spores of *A. alternata* to 60°C for about 15 s in vitro reduced germination by 48 percent (Fallik et al., 2000).

The survival of fungal spores exposed to solutions containing up to 30 percent (v/v) ethanol at 25–50°C for 30 s was determined by germination on semisolid media. Logistical, second-order surface-response models were prepared for each fungus. Subinhibitory ethanol concentrations at ambient temperatures became inhibitory when heated at temperatures much lower than those that cause thermal destruction of the spores by water alone. At 40°C, the estimated ethanol concentrations that inhibited the germination of 50 percent ( $LD_{50}$ ) of the spores of *A. alternata* and *Aspergillus niger* were 14 and 20 percent, respectively (Gabler et al., 2004).

The kinetics of the conidia survival of pathogenic *Aspergillus* species, like *A. fumigatus*, *A. flavus*, and *A. niger*, following exposure to heating at 60°C and microwave irradiation was studied by Araujo et al. (2006). Heating the conidia of *A. flavus* and *A. niger* at 60°C for 45 min was found to be fungicidal (reduction  $> 10^4$  conidia/ml), but was not effective against *A. fumigatus* conidia.

Short periods of microwave irradiation (40 s) resulted in a significant reduction of the viability of the conidia of these three *Aspergillus* species as a result of lethal membrane lesions. Growth of *P. expansum* was completely inhibited by a 2450 MHz microwave heating for 2 or 3 min. The population density of *P. expansum* in surface wounds of fruit treated with microwave for 2–3 min was significantly lower than that of the untreated control (Zhang et al., 2006). The combined effect of simultaneous application of heat treatments, and low frequency ultrasound (20 kHz) at different amplitudes, on *A. flavus* spore viability suspended in laboratory broth formulated at different  $a_w$  (0.99 or 0.95) and pH (5.5 or 3.0), with or without vanillin or potassium sorbate, was evaluated (Lopez-Malo et al., 2005). When combining temperatures of 57.5 or 60°C, high ultrasound amplitudes and the presence of potassium sorbate or vanillin, very short treatments were found to be sufficient to eliminate the initial inoculated spores of *A. flavus*.

A study was performed to evaluate the antifungal activity of seven *Allium* plants against *A. niger*, *A. flavus*, and *A. fumigatus*. Although heat treatments (65 or 100°C for 30 min) decreased the inhibitory effect for most *Allium* plants, the presence of acetic acid significantly improved the adverse influence of heat in this study (Yin and Tsao, 1999).

Hot-water treatment for durations of up to 60 min at 50°C failed to reduce the incidence of recovery of seedborne *A. niger* and *A. flavus* delivered from seeds incubated at 30°C on agar; *A. fumigatus* was not recovered from seeds treated in this way. However, when seeds were hot-water treated at 60°C and incubated on agar at 30°C, *A. niger* was virtually eliminated by a treatment duration of 15 min or more; the incidence of recovery of *A. fumigatus* was significantly increased compared with the 50°C treatment and there was no change in the incidence of *A. flavus*. Hot-water treatment at 60°C for more than 30 min significantly reduced seed germination (Hayden and Maude, 1994).

### 15.3. IN VIVO

To maximize control of fruit decay by alternatives to synthetic fungicides after harvest, various control strategies can be integrated. Inhibition of physiological or/and pathological deterioration of fresh harvested produce was studied by a combination of any type of physical, chemical, or environmentally friendly chemical methods called “physicochemical” treatments (Table 15.1).

#### 15.3.1. HEAT TREATMENTS OF DRY AND FEED PRODUCTS

Attention has been drawn to mycotoxin-producing fungi which naturally contaminant cereals, and their harmful toxins. Heat treatment at 64°C by drum drying does not eliminate already formed mycotoxins but the technique can reduce the number of viable fungi on the grain. Drum drying very efficiently reduced the fungal propagules colonizing the grain, including the

TABLE 15.1 The optimal temperature and time of exposure of heat treatments, alone or in combination with other treatments, to inhibit or eliminate decay development on fresh and dry commodities.

Crop	Heat treatment HAT <sup>a</sup> , HWD <sup>b</sup> ; HWRB <sup>c</sup>	Optimal temperature/ time	Fungus–pathogen	Reference
Apple	HAT+Ca <sup>d</sup> + antagonist	38°C/4 days	<i>Penicillium expansum</i>	Conway et al., 2004, 2005
Apple	HWRB	55°C/15 s	<i>P. expansum</i>	Fallik et al., 2001
Apple	HAT+antagonist	38°C/4 days	<i>P. expansum</i>	Leverentz et al., 2000, 2003
Apple	HAT+antagonist+1-MCP <sup>e</sup>	38°C/4 days	<i>P. expansum</i>	Janisiewicz et al., 2003
Apple	HAT+1-MCP+CA <sup>f</sup>	38°C/4 days	<i>P. expansum</i>	Saftner et al., 2003
Grain	HAT (dry heat)	64°C	<i>Penicillium</i> spp./ <i>Fusarium</i> spp.	Kristensen et al., 2005
Litchi	HWRB	55°C/20 s	<i>Penicillium</i> spp.	Lichter et al., 2000
Maize	HAT (dry heat)	60–70°C	<i>F. verticilloides/Aspergillus flavus</i>	Hawkins et al., 2005
Mango	HWRB+Prochloraz <sup>g</sup>	48–62°C/20 s	<i>Alternaria alternata</i>	Prusky et al., 1999, 2002
Mango	HWD or HAT	50°C/5 min or 40°C/4 h	<i>A. alternata</i>	Mansour et al., 2006
Melon (Galia type)	HWRB	59°C/15 s	<i>A. alternata/ Fusarium</i> spp.	Fallik et al., 2000
Onion	HAT+fungicide	35°C/8 h	<i>Aspersillus porri</i>	Sugha et al., 1993
Peach and nectarine	HWRB+ antagonist	60°C/20 s	<i>P. expansum</i>	Karabulut et al., 2002
Peach	HWD+MAP+antagonist	55°C/10 s	<i>P. expansum</i>	Karabulut and Baykal 2004
Peanuts	HAT (dry heat)+ozone	75°C/15 min	–	Proctor et al., 2004
Pear	HWRB+wetting agent	30°C	<i>P. expansum</i>	Bai et al., 2006; Spotts et al., 2006
Pear	Microwave+antagonist		<i>P. expansum</i>	Zhang et al., 2006
Pepper (chilli)	HWD	52°C/15 min	<i>Aspergillus</i> spp.	Ajithkumar and Naik, 2006
Pepper (sweet)	HWD	50°C/3 min	<i>A. alternata</i>	Fallik et al., 1996
Pepper (sweet)	HWRB	55°C/12 s	<i>A. alternata</i>	Fallik et al., 1999
Rock melon	HWD	60°C/1 min	<i>Fusarium</i> spp., <i>Alternaria</i> spp.	McDonald et al., 2005
Sweet cherry	HWD+ethanol (10%)	60°C/3 min	<i>P. expansum</i>	Karabulut et al., 2004

<sup>a</sup>HAT – Hot air treatment (dry or forced, or vapor, or curing)

<sup>b</sup>HWD – Hot-water dip

<sup>c</sup>HWRB – Hot-water rinsing and brushing

<sup>d</sup>Ca – Calcium

<sup>e</sup>1-MCP – 1-methycyclopropene

<sup>f</sup>CA – Controlled atmosphere

<sup>g</sup>Fungicides



mycotoxin-producing *P. verrucosum*, *Fusarium avenaceum*, *F. culmorum*, *F. poae*, *F. sporotrichoides*, and *F. tricinctum*. A similar drying regime in a supplementary trial was found to reduce the number of *P. verrucosum* contaminated kernels from more than 70 to 12 percent but confirmed that drum drying did not destroy already formed ochratoxin A (Kristensen et al., 2005). After harvest, maize is dried artificially to halt fungal growth and mycotoxin production while in postharvest storage. The process often limits harvest capacity and has been a frequent cause of seed injury. Higher drying temperatures could lead to shorter drying periods and faster turnover; however, there is often a deterioration of the physical grain quality, including increased breakage susceptibility and loss of viability. At temperatures above 60°C, *F. verticilloides* kernel infection was significantly reduced to less than 18 percent. At 70°C, there was a significant reduction in *A. flavus* kernel infection, from 11 to 3 percent (Hawkins et al., 2005). The study was conducted to evaluate the effectiveness of ozonation and mild heat in breaking down aflatoxins in peanut kernels (Proctor et al., 2004). Peanuts were subjected to gaseous ozonation and under various temperatures (25, 50, 75°C) and exposure times (5, 10, 15 min). Ozonation efficiency increased with higher temperatures and longer treatment times. However, the temperature effect lessened as the exposure time increased, suggesting that ozonation at room temperature for 10–15 min could yield degradation levels similar to those achieved at higher temperatures while being more economical.

Gowda et al. (2007) reported that drying of feed either in hot air oven (80°C, 6 h) or in sunlight (14 h for 2 days, 25–37°C ambient temperature) is effective in reducing the aflatoxin level and the harmful effects in sheep; the latter method of treatment is more effective and practical, and is less expensive.

### 15.3.2. HOT-WATER RINSING AND BRUSHING (HWRB)

HWRB technology applied for decay reduction in fruits and vegetables has been reviewed recently by Fallik (2004). The produce is exposed to water at temperatures between 48 and 63°C for 10–25 s while rolling over brushes, depending upon produce type and cultivar. At the end of the hot-washing treatment, forced-air fans or hot forced-air is used to dry the produce inside a tunnel for 1–2 min (Fallik, 2004). HWRB was tested on “Galia” type melon (*Cucumis melo* cv. *reticulatus*) fruit. The optimal treatment to reduce decay caused by *Alternaria alternata* and *Fusarium solani*, while maintaining fruit quality after prolonged storage and marketing simulation, was  $59 \pm 1^\circ\text{C}$  for 15 s. Trial shipments by sea transport to Europe demonstrated that treating melon with a commercial-scale HWRB machine maintained significantly better overall quality of treated fruit compared with untreated fruit (Fallik et al., 2000). Deciduous fruits were found to benefit from HWRB treatments as well. HWRB at 55°C for 15 s significantly reduced decay development in *P. expansum*-inoculated apple fruit after 4 weeks at 20°C, or in naturally infected (*P. expansum*) apple fruit after prolonged storage of 4 months at 1°C

plus 10 days at 20°C (Fallik et al., 2001). Recently Bai et al. (2006) and Spotts et al. (2006) reported that treating d'Anjou' pears (*Pyrus communis* L.) with a washing system with high-pressure spray, warm water (30°C), and wetting agent over rotating soft brushes was significantly effective in decay control without causing internal or external damage of fruit. Reductions in *P. expansum* decay by this system reached 13 percent.

### 15.3.3. HOT-WATER DIPS (HWD)

Most hot-water dip treatment facilities that are in commercial use are of the batch system, or the continuous system. This is a medium range treatment that lasts several minutes. Curing is considered a moderate heat treatment and lasts for hours.

Hot-water dipping (HWD) treatment at 52°C for 15 min showed 100 percent inhibition of *Aspergillus* spp. growth on chilli pepper fruits (Ajithkumar and Naik, 2006). Dipping naturally infected or artificially inoculated sweet pepper fruit at 50°C for 3 min significantly reduced decay development caused by *A. alternata* (Fallik et al., 1996).

Dipping rock melons in hot water at 50, 55, 60, and 65°C for 1 min inhibited disease development caused by *Fusarium* spp. and *Alternaria* spp. at all the temperatures in comparison to the control (22°C). However, a 1-min exposure to 60°C was the optimum temperature, resulting in 97 percent of fruit being disease-free after 3 weeks storage at 5°C compared to only 7 percent when dipped in 22°C water (McDonald et al., 2005). Mansour et al. (2006) reported that dipping mango fruits in hot water at 50°C for 5 min or holding in hot air (HA) for 4 h at 40°C was the most effective treatment for retarding postharvest disease caused by *A. alternata*, without peel blackening and fruit damage. These heat treatments increased the shelf life of inoculated and uninoculated fruits.

### 15.2.4. USING HEAT TREATMENT AND ANTAGONISTIC MICROORGANISMS

Different strategies have been proposed as alternatives to fungicides to control postharvest diseases on fruits and vegetables. Utilization of antagonistic microorganisms together with physical treatments appears to be a promising technology. Some antagonist-based products are commercially available and other are currently at varying stages of development (De Costa and Erabadupitiya, 2005).

A combination of heat (38°C for 4 days) and the antagonist *Pseudomonas syringae*, or one of two yeast antagonists, was more effective than either treatment alone in controlling decay development caused by *P. expansum* on apples, even when there was a delay of the heat treatment for up to 24 h

(Leverentz et al., 2000, 2003). The combination of these two control measures, therefore, was complementary and resulted in better decay control than either treatment alone.

In vivo test, microwave power and antagonistic yeast, as stand-alone treatments of pears, were capable of reducing the percentage of infected wounds from 100 to 72.6 and 65.5 percent, and lesion diameter from 2.81 to 2.20 and 1.85 mm, respectively. However, in fruit treated with combination of microwave power and the antagonist *Cryptococcus laurentii*, the percentage of infected wounds and lesion diameter was only 20.2 percent and 1.1 mm, respectively, while incidence of natural decay on treated fruits were similar to that of inoculated fruit. None of the treatments did impair quality parameters of fruit. Thus, the combination of microwave and *C. laurentii* could be an alternative to chemicals for the control of postharvest blue mold rot on pear fruits (Zhang et al., 2006).

### 15.2.5. HEAT TREATMENTS IN COMBINATION WITH OTHER PHYSICOCHEMICAL MEANS

Several combinations of physicochemical treatments to improve the efficacy of heat treatments to control decay development were reported.

Saftner et al. (2003) reported that prestorage application of 1-methylcyclopropene (1-MCP), heat (38°C for 4 d), 1-MCP plus heat treatments and controlled atmosphere (CA) storage decreased decay severity caused by wound-inoculated “Golden Delicious” apples [*Malus sylvestris* (L.) Mill] by *P. expansum* Link. However, poststorage of 1-MCP treatment had no effect on decay severity. An antagonist, *Metchnikowia pulcherrima* T5-A2, was used in combination with heat (38°C for 4 days) and 1-MCP treatments to control the blue mold caused by *P. expansum* on “Golden Delicious” apples under controlled atmosphere (CA) conditions for 4 months (Janisiewicz et al., 2003). “Golden Delicious” apples were wound-inoculated with *P. expansum* and then treated with various combinations of heat (38°C) for 4 days, 2 percent sodium bicarbonate, and two biocontrol agents alone or combined. All the treatments that included heat virtually eliminated decay caused by *Penicillium* (Conway et al., 2004, 2005). Heat treatment and sodium bicarbonate significantly improved the efficacy of the biocontrol agent (BIO126 strain of *M. pulcherrima*) against blue mold on apple fruit stored at 23°C (Spadaro et al., 2004).

Several combinations of physicochemical treatments were studied in order to improve the efficacy of HWRB to control decay development. A combined HWRB treatment at 48–62°C, depending on the cultivar, for 15–20 s with 225 mg ml<sup>-1</sup> prochloraz was the most effective treatment for control of *Alternaria* rot in mango fruit with a high level of quiescent surface infection (Prusky et al., 1999, 2002). HWRB at 55°C for 20 s significantly reduced chemical use (prochloraz) to control decay development caused mainly by *Penicillium* spp. in litchi fruit after 5 weeks storage and a marketing simulation period (Lichter

et al., 2000). Treating nectarine and peach fruits with HWRB at 60°C for 20 s and dipping them in a cell suspension ( $10^8$  cells per ml) of *Candida* spp. 24 h after inoculation with *P. expansum* reduced decay development by 60 percent compared with the controls (Karabulut et al., 2002).

Ethanol (EtOH) occurs naturally in fruits and many other food products, and its toxicological properties on fungal pathogens have been studied (Margosan et al., 1997). Karabulut et al. (2004) reported that immersing sweet cherry in hot water at 50, 55, or 60°C alone was not effective against *P. expansum*, while their efficacies were significantly increased by the addition of 10 percent ethanol. The most effective treatment was immersion in 10 percent ethanol at 60°C. Ethanol treatments at 20, 30, 40, or 50 percent and water treatments at 55 or 60°C significantly reduced natural fungal populations on the surfaces of fruit in all of the experiments.

An integrated approach was studied for the control of postharvest diseases of peaches including application of a yeast antagonist (*Candida oleophila*), hot-water treatment at 55°C for 10 s, and storage in modified atmosphere at 0°C (Karabulut and Baykal, 2004). Hot water and the yeast antagonist as stand-alone treatments were not effective in controlling *P. expansum* infections on wound-inoculated peaches. However, all of the treatments significantly reduced the decay incidence caused by natural infection. The highest efficacy was achieved by the combination of all three tactics.

The effect of heat treatment on bulbs alone, or in combination with a fungicidal spray to control of purple blotch [*Alternaria porri* (Ellis) Cif.] of onion (*Allium cepa* L.) was reported by Sugha et al. (1993). Heat treatment to onion bulbs at 35°C for 8 h before sowing followed by single prophylactic spray of fungicides at bolting stage or only three sprays of the fungicides at 15-day intervals (from the appearance of disease) gave the most effective control of purple-blotch disease. Heating of bulbs at 40 and 45°C reduced the crop growth.

## 15.4. CONCLUSIONS

Mycotoxins that have been associated with severe toxic effects to vertebrates are produced by many important phytopathogenic and food spoilage fungi including *Aspergillus*, *Penicillium*, and *Alternaria* species (Kabak et al., 2006).

Recent studies in Europe through a cluster of research projects have been carried out within the framework of Hazard Analysis Critical Control Point (HACCP) approaches to identify critical control points (CCPs) in these food production processes. Within a HACCP system for mycotoxin control, CCPs are key stages in the production where control can and needs to be maintained to give safe product with respect to trichothecene and ochratoxin contamination (Magan, 2006).

Several control strategies to prevent the growth of mycotoxigenic fungi as well as to inhibit mycotoxin biosynthesis including preharvest (resistance varieties, field management, and the use of biological and chemical agents),

harvest management, and postharvest (improving of drying and storage conditions, the use of natural and chemical agents, physical treatments, irradiation, and combined treatments) applications have been reviewed recently (Kabak et al., 2006). While much work in this area has been performed on aflatoxin B-1 and ochratoxin A, much less information is available on other mycotoxins such as patulin and citrinin. In addition, physical, chemical, and biological detoxification methods used to prevent exposure to the toxic and carcinogenic effect of mycotoxins were mentioned. Moreover, recent work has taken an alternative view by looking at the potential of using antioxidants, essential oils from plants and other natural products from bacteria and fungi. These studies have shown that some essential oils such as cinnamon and clove leaf oil have the capacity for control of *P. verrucosum*, *A. ochraceus* or *Fusarium* species and their mycotoxins (including ochratoxin A) production depending on the environmental conditions (Cairns and Magan, 2003; Hope et al., 2003). However, there are many economic and technological hurdles associated with this type of approach. Resveratrol, a naturally occurring phytoalexin, is produced by several higher plants in response to injury or fungal infection. A number of beneficial health effects, such as anti-cancer, anti-viral, neuroprotective, anti-aging, anti-inflammatory, and life-prolonging effects have been reported (Fremont, 2000). Resveratrol has been demonstrated to have a particularly wide spectrum of mycotoxin control, although at present this is a relatively expensive product (Fanelli et al., 2003). Finally, dietary strategies which are one of the most recent approaches to counteract the mycotoxin problem with special emphasis on in vivo and in vitro efficacy of several binding agents (activated carbons, hydrated sodium calcium aluminosilicate, bentonite, zeolites, and lactic acid bacteria) have also been reviewed by Kabak et al. (2006).

The review highlighted here has considered some key aspects of prevention strategies which require the integration of scientific knowledge and effective management of the food production chain to prevent mycotoxin contamination. The identification of key CCPs and detailed information on the ecophysiological conditions which allow toxin production are key to determining the monitoring points and methods to be used (Kabak et al., 2006). This information when combined with some of the diagnostic tools detailed above enable the integration of climatic data, production processes, and diagnostic systems to develop effective DSS. These can then be used to predict whether there is a high or low risk from mycotoxin contamination in relation to key mycotoxins for which regulatory limits exist. This will contribute significantly to minimizing consumer exposure to such natural toxic compounds.

## ACKNOWLEDGMENT

This is a contribution from the Agricultural Research Organization, the Volcani Center, Bet Dagan, Israel No. 482/07.

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# Biological Control of Mycotoxigenic Fungi in Fruits

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## 16.1. INTRODUCTION

Mycotoxins are toxic metabolites produced by fungi. The ability to produce mycotoxins is found in some fungal pathogens of plants and/or moulding agents of food and feed, and has a noteworthy repercussion on the quality and safety of food products. Mycotoxin contaminations now present one of the most insidious challenges to meet in food safety. Their importance is evident in the constant attention paid to them internationally, because of their harmful impact on animal and human health as well as on the economy. The most prevalent toxigenic fungi on fresh fruits belong to the genera *Aspergillus*, *Penicillium* and *Alternaria*, which pose serious mycotoxicological risks essentially in postharvest and processed food products. Although the ability of these fungi to produce and contaminate fresh and processed fruit with mycotoxins is well established, mostly they are referred to in the pathological context as rot-causing pathogens. In this regard, it is necessary to discuss not only postharvest losses attributed to these pathogens, but also the toxic metabolites they produce.

The use of microbial antagonists for the control of postharvest pathogens, including mycotoxigenic fungi, will be discussed in detail, taking into account the commercial potential and perspectives of the practical application of these biological control agents and their mechanisms of action. Also the pathogenicity factors of postharvest pathogens and the possible role of mycotoxins are presented, with regard to their possible influence on and interaction with the biocontrol agents. Other approaches to the control of postharvest pathogens such as physical control (e.g., controlled atmosphere, heat treatment) and chemical control are presented in separate chapters of this book. Their integration

with microbial antagonists should also be taken into account. In this chapter, however, integration of biocontrol agents with chemical control *only* is discussed, while combination of these beneficial microorganisms with controlled atmospheres is not mentioned because it is still in its infancy.

## 16.2. BIOLOGICAL CONTROL OF FUNGAL PATHOGENS IN FRUITS

The use of epiphytic microorganisms for the control of postharvest pathogens of fruits has been rapidly evolving during the last two decades. In Cook and Baker's book on biocontrol (Cook and Baker, 1983), only one example of the biocontrol of postharvest disease of a fruit was presented. This was the work of Tronsmo and Dennis (1977), in which *Trichoderma* was used to control *Botrytis* rot of strawberry. Subsequently, Wilson and Pusey (1985) presented their ideas on the potential of postharvest biocontrol in a feature article and reported their initial research on using a strain of *Bacillus subtilis* to control brown rot on peach, caused by *Monilinia fructicola*. This work highlighted the viability of this approach and gave prospects for further development and commercial use. Since that time, research on biocontrol of postharvest diseases has been growing rapidly and has been the subject of numerous scientific meetings, review articles and books that provided a chronological account of the scientific advances that have been made in postharvest biocontrol, as well as the problems faced in trying to develop a commercial product (Wilson and Wisniewski, 1989, 1994; Wilson et al., 1996; Droby et al., 2000, 2001; Wisniewski et al., 2001; El Ghaouth et al., 2002, 2004).

Initially, the basic rationale underlying research efforts in this field was to reduce the use of synthetic chemicals on harvested commodities and the environmental contamination caused by these compounds. This motivation was strengthened by a U.S. National Academy of Sciences (NAS) report of 1987 (U.S. National Research Council, Board of Agriculture, 1987) that stated, "As a class, fungicides present special difficulties because nine oncogenic compounds account for about 90 percent of all fungicide sales." This report indicated that fungicides collectively make up 60 percent of the oncogenic risk among all pesticides. Furthermore, it concluded that the discontinued use of these chemicals would have an adverse economic impact on the production of some crops because of the dearth of viable alternatives. This issue heightened the concerns about the consequences associated with the decertification of some of the fungicides used to manage postharvest disease. The potential health risk associated with fungicides has also previously been highlighted by a report by NAS (U.S. National Research Council, Board of Agriculture, 1983) that documented the increased vulnerability of children to synthetic pesticides. Reports of the development of resistance to fungicides (Spotts and Cervantes, 1986) also helped to establish the need to develop new, effective alternatives for managing postharvest diseases that posed less risk to human

health and the environment than current practices of using synthetic fungicides. Lastly, environmental preservation from chemicals is becoming increasingly important because of present climate changes that are taking place as a consequence of global warming. These changes are likely to cause water shortage in many areas of the world and, as a consequence, a potential increase in the concentration of contaminants in the environment.

### 16.2.1. DEVELOPMENT OF POSTHARVEST BIOCONTROL PRODUCTS

Biological control as an approach to managing plant disease has had a limited number of major, commercial success stories. One of the major challenges in applying biocontrol agents in the field is that environmental conditions can profoundly affect their survival and effectiveness. In the postharvest environment, parameters such as temperature and humidity are rigidly controlled and can be taken into account when selecting a suitable biocontrol agent. Also, harvested commodities present a more concentrated target for the application of biocontrol agents. The regulated environment, the ability to target the application of the biocontrol agent, and the high value of the harvested commodity together suggested that the use of biocontrol agents to manage postharvest disease should have an excellent chance of success (Gueldner et al., 1988).

By the early 2000s, there were three postharvest biological products available on the market: Aspire<sup>TM</sup> (limited to the United States and Israel), BIOSAVE<sup>TM</sup> (limited to the United States) and YieldPlus<sup>TM</sup> (limited to South Africa). Other products (Biocure, Biocoat) were developed, but have not yet reached the market place. They are based on a combination of natural products along with a yeast antagonist to address the poor ability of other postharvest biocontrol products to control pre-established and latent infections. The main components of these products are the yeast antagonist, *Candida saitoana*, and either a derivative of chitosan (El Ghaouth et al., 2000) (Biocoat) or lysozyme (Biocure). Both products contain additional additives, such as sodium bicarbonate, to enhance efficacy and to perform as well as currently available postharvest fungicides. Another recently developed product has taken the approach of preventing postharvest decay with the application of a yeast biocontrol agent applied to flowers and fruits in the field several times throughout the growing period. This approach also addresses the problem of pre-established or latent infections. The product is based on the use of a heat-tolerant strain of *Metschnikowia fructicola* and is marketed under the name SHEMER<sup>TM</sup> in Israel by the company AgroGreen, Ashdod. It has been shown to be effective against rots caused by *Botrytis*, *Penicillium*, *Rhizopus* and *Aspergillus* on strawberries, grapes and citrus (Kurtzman and Droby, 2001; Karabulut et al., 2003, 2004).

In spite of all the published basic and applied research on postharvest biocontrol, the commercial use of these products has been limited and accounts for only a very small fraction of the potential market. As discussed in several

reviews (Droby et al., 2000, 2001; Wisniewski et al., 2001; El Ghaouth et al., 2002, 2004), the main shortcoming with the use of postharvest biocontrol products has been inconsistency in performance, especially when used as a stand-alone product in place of synthetic fungicides. A second problem with the products that are currently available is their inability to control previously established and latent infections. The reasons for these shortcomings have been reviewed by Droby et al. (2001). In short, the following factors can dramatically affect the viability and performance of postharvest biocontrol agents: fermentation and formulation practices; the method by which the product is delivered to the commodity; inoculum pressure; and the physiological status of the fruit. Additionally, the strategy used to identify potential antagonists has favoured the selection of organisms that exhibit protective rather than eradicated activity (El Ghaouth et al., 2004).

### 16.3. BIOLOGICAL CONTROL OF PATULIN AND OCHRATOXIN A-PRODUCING FUNGI

Patulin and ochratoxin A are mycotoxins that contaminate fruits and fruit-derived products. The highest tolerable levels of these toxins have been established in many countries. Fruit-derived products that can be contaminated are pome fruit-based juices and baby food for patulin, and wine, raisins and grape juice for ochratoxin A (Castoria and Logrieco, 2007).

The occurrence of ochratoxin A in raw agricultural products has been amply documented worldwide in a variety of plant products, and has also been reported recently in wine and grape juice (Zimmerli and Dick, 1996; Visconti et al., 1999; Pietri et al., 2001). Black aspergilli, and primarily strains of *Aspergillus carbonarius* and representatives of the *Aspergillus niger* aggregate are considered the causes of ochratoxin A contamination in grapes and grape-derived products following attack in the field and during storage (Abarca et al., 2001; Battilani and Pietri, 2002; Cabanès et al., 2002; Da Rocha Rosa et al., 2002; Magnoli et al., 2003; Battilani et al., 2004). Available evidence indicates that these black aspergilli are mainly saprophytes and are responsible for secondary rot (Pitt and Hocking, 1997). Research aimed at developing biocontrol strategies, in order to reduce the presence of ochratoxigenic fungi in various commodities, has been carried out by many scientists around the world (Bhatnagar et al., 2002).

Biological control as part of Integrated Pest Management (IPM) programmes against *Aspergillus* and *Penicillium* bunch rot in grapes has been tested in vineyards and after harvest by several research groups. The efficacy of microbial antagonists in combination with reduced rates of fungicide applications was evaluated. Among the organisms reported to be effective were *Trichoderma harzianum* (Elad, 1994), *Aureobasidium pullulans* (Lima et al., 1997), *Pythium periplocum* (Paul, 1999), *Metschnikowia fructicola* (Karabulut et al., 2003), various yeasts (Wilson et al., 1991; Zahavi et al., 2000) and *Bacillus subtilis* (Pusey, 1989). Currently, no single biocontrol agent seems to be sufficiently effective to

alone suppress bunch rot of grapes before and after harvest. The balance in the future may, however, shift in favour of biocontrol with the support of IPM programs. Recently, experiments carried out at laboratory level showed that some *A. pullulans* strains are highly suppressive to rot caused by *A. carbonarius* on detached and wounded wine grape berries, i.e., under conditions that are favourable to the fungal pathogen. Interestingly, these strains are able to degrade ochratoxin A to the less toxic compound ochratoxin  $\alpha$  in vitro. Furthermore, in the few berries that were infected by the fungus and were pretreated with the biocontrol agents, ochratoxin A contamination was lower than in infected non-treated berries (De Felice et al., 2007). Ochratoxin  $\alpha$  was also detected, but its origin could not be ascertained. It could, in fact, have originated from degradative or biosynthetic activity of *A. carbonarius*, since mycotoxin-producing fungi themselves are able, under certain conditions, to degrade the toxins that they produce (Karlovsy, 1999; Bejaoui et al., 2006), and ochratoxin  $\alpha$  is also a precursor in ochratoxin A biosynthesis (Harris and Mantle, 2001).

Several reports on biological control of the patulin-producing fungus *Penicillium expansum* in apples exist in the scientific literature, whereas, to our knowledge, no papers have yet been published on biological control of ochratoxin A-producing fungi on wine grape. Therefore, the following section will focus on the experience accumulated on biological control of *P. expansum* and the theoretical and practical implications of the biocontrol of this fungus.

### 16.3.1. BIOLOGICAL CONTROL OF PATULIN-PRODUCING *PENICILLIUM EXPANSUM* ON STORED POME FRUITS

Patulin is a mycotoxin produced by *P. expansum*. It contaminates pome fruits and derived products as a consequence of the infection of the fruits by the mycotoxigenic fungus, resulting in blue mould of apples and pears. Although the infection by *P. expansum* can take place through the stem calyx, the main penetration sites of this fungus are superficial wounds caused during harvesting, transportation and by improper handling.

Patulin is an unsaturated lactone (4-hydroxy-4H-furo[3,2c]pyran-2(6H)-one) (Fig. 16.1) that the International Agency for Research on Cancer has

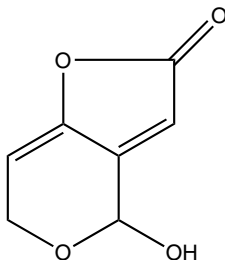


FIGURE 16.1 Chemical structure of patulin.

placed in group 3, i.e., with compounds that are unclassifiable as to carcinogenicity in humans. However, some reports indicate that this mycotoxin exerts *in vitro* mutagenic and genotoxic effects and causes oxidative damage to the DNA of mammalian cells, such as human peripheral blood lymphocytes and embryonic kidney cells (Biing-Hui et al., 2003; Schumacher et al., 2005).

At the cellular level, patulin appears to induce oxidative stress by lowering the concentration of the antioxidant peptide glutathione, to which it binds due to its electrophilic reactivity, and through generation of reactive oxygen species (Barhoumi and Burghardt, 1996; Fliege and Metzler, 2000; Rychlik et al., 2004). Patulin also has immunotoxic and immunosuppressive effects (Bourdiol et al., 1990; Wichmann et al., 2002). The utilization of decaying and contaminated apples for the production of fruit juice is the reason why contamination of this product with patulin has frequently been detected (Beretta et al., 2000; Leggott and Shephard 2001; Yurdun et al., 2001; Martins et al., 2002; Tangni et al., 2003; Ritieni, 2003; Piemontese et al., 2005; Castoria and Logrieco, 2007). Since children consume great quantities of fruit juices, many countries have established the highest tolerable levels of patulin in juices and baby foods (Castoria and Logrieco, 2007).

Generally speaking, biological control of *P. expansum* based on antagonistic microbes does not imply different approaches as compared to the biocontrol of other major postharvest wound pathogens such as *Botrytis cinerea*; the main goal is the prevention of the attack by the mycotoxigenic fungus on stored pome fruits, in particular apples, because once the infection has taken place, patulin synthesis and fruit contamination also occur. Although primarily the targeted pathogen should be considered when developing a biological control strategy, biocontrol of wound pathogens affecting stored fruits should be effective in the prevention of a wide range of diseases in order to make this approach more viable from an economic point of view.

Several antagonistic microbes have been successfully used for preventing infections by postharvest fungal pathogens of pome fruits, including *P. expansum* (Janisiewicz and Korsten, 2002). Some formulations based on antagonistic bacterial and yeast strains have reached the commercialization stage (see Section 16.2).

The activity of postharvest biocontrol agents relies on a variety of complex mechanisms (Droby and Chalutz, 1994; Janisiewicz and Korsten, 2002; Castoria et al., 2003), which make the selection of resistant strains of the pathogen unlikely. Conversely, this selection takes place when the few authorized postharvest fungicides are used, especially those acting on fungal targets coded by one or a few genes, as in the case of benzimidazoles and  $\beta$ -tubulin (Sholberg et al., 2005).

As mentioned in section 16.2, the implementation of biological control is needed to reduce the utilization of chemical fungicides that can reach the consumer's table, and to lower environmental contamination with these compounds. Nevertheless, the utilization of chemicals – at a reduced dosage or number of applications – combined with biocontrol agents that are compatible with (i.e., resist) fungicides is worth considering and exploring. Integrated

control has actually proven to be very effective in preventing postharvest diseases (Lima and De Cicco, 2006), and can also control fungicide-sensitive and fungicide-resistant mixed pathogen populations (Lima et al., 2006). The onset of fungicide-resistant populations of fungal pathogens has been reported for benzimidazole-based control of mycotoxigenic *P. expansum*, in most cases as the consequence of a single base substitution at the codons 198 and 200 of the  $\beta$ -tubulin gene sequence (Sholberg et al., 2005). The integrated approach probably exerts a weaker selection pressure, thus lowering the probability of the onset of fungicide-resistant populations of fungal pathogens.

Whatever the approach used to control mycotoxigenic *P. expansum* on pome fruits, infections cannot be completely prevented, and as a consequence, patulin contamination cannot be set to zero. In this regard, the utilization of biocontrol agents could definitely represent a promising tool, if the results reported by Castoria et al. (2005) on a laboratory scale (discussed in Section 16.3.2), are confirmed on a commercial scale (as well as with other biocontrol agents), and the mechanisms underlying such results are elucidated and, possibly, exploited.

### 16.3.2. INFLUENCE OF A BIOCONTROL YEAST ON PATULIN ACCUMULATION IN STORED APPLES: A CASE STUDY

The impossibility of completely preventing *P. expansum* infection and patulin contamination on stored apples led Castoria et al. (2005) to examine the influence of the biocontrol yeast *Rhodotorula glutinis* strain LS11 on the accumulation of patulin in apples infected by the fungus. This basidiomycetous yeast strain had already proved effective in preventing fungal attack on pome fruits in semi-commercial conditions, also when applied at a reduced rate (10 percent of the normally applied full dose) of benzimidazolic fungicide (Lima et al., 2003). In their study, Castoria et al. (2005) reported that in apples pretreated with the biocontrol agent, the overall accumulation of patulin in the low percentage of *P. expansum*-infected fruits was significantly lower than in infected, non-pretreated apples. Furthermore, the biocontrol yeast was able to resist and degrade a high concentration of patulin in vitro and in a model system consisting of browning apple homogenate, which mimicked decaying fruit tissue. One of the major issues to be considered in the biodegradation of mycotoxins is that degradation products are less toxic (or possibly not toxic at all) than the original mycotoxin (Karlovsy, 1999). Interestingly, the major patulin degradation product formed in vitro by *R. glutinis* LS11 characterized by NMR analyses was identified as desoxypatulinic acid (Castoria et al., 2006), which was reported to be non-toxic to different microorganisms that are inhibited by patulin (Scott et al., 1972).

The measurement of patulin degradation in vivo (i.e., in *P. expansum*-infected apples pretreated with the biocontrol yeast) by analyzing the content of desoxypatulinic acid has yet to be carried out. The biocontrol yeast cells survive on the surface of infected apple wounds, but do not reach the deeper



internal layers of the decaying fruit tissue, where the core of patulin synthesis presumably occurs (Castoria et al., 2006). The latter observation suggests that patulin degradation by *R. glutinis* LS11 may not occur under these conditions. However, this mechanism cannot be ruled out, since patulin diffusion from fungus-infected apple tissue to healthy areas of the fruit has been reported (Beretta et al., 2000; Rychlik and Schieberle, 2001), and therefore, at least some toxin could reach the yeast cells. The reduction of patulin contamination could also be due to inhibited development of fungal mycelium in the infected apples caused by the biocontrol agent, as suggested by the reduction of lesion diameters (Castoria et al., 2005). However, lesion diameters can be considered only as a semi-quantitative index of pathogen growth in the decaying fruit tissue. Xu and Berrie (2005) showed that in most of the apple cultivars that are more resistant (i.e., develop smaller lesions) to infection by an aggressive isolate of *P. expansum*, the patulin accumulation is higher than in cultivars developing more severe symptoms. This suggests that patulin synthesis could be enhanced as a fungal response to stressful conditions (higher apple resistance). Competition among microorganisms for essential environmental factors, such as nutrients and space, is a stressful condition and is expected to have a dramatic effect on the secondary metabolism of spoilage moulds. Nutrient availability strongly affects secondary metabolism, including mycotoxin production (Luchese and Harrigan, 1993). Actually, in a stressful condition such as competition between different *Fusarium* spp. when co-inoculated on their host plant, mycotoxin production, as measured per unit of fungal biomass basis through real-time PCR, was much greater than in single inoculation experiments (Xu et al., 2007). Therefore, also in the case of the interaction between the biocontrol agent LS11 and *P. expansum* on apples, the relationship between fungal biomass and patulin synthesis should be assessed through quantitative real-time PCR, which is now possible since species-specific sequences are available (Paterson et al., 2003).

Whatever the mechanism involved in the reduction of patulin contamination in biocontrol yeast-treated apples infected by *P. expansum*, this finding needs to be confirmed on a commercial scale for LS11, as well as for other biocontrol agents. Nevertheless, it encourages further research on microbial biocontrol as a possible strategy for reducing mycotoxin contamination on fruit crops. The identification of enzyme(s)/gene(s) responsible for toxin conversion could pave the way for the development of new technologies for the prevention and/or detoxification of patulin contamination in pome fruit-based products. Moss and Long (2002) reported the anaerobic metabolization of patulin by the ascomycetous yeast *Saccharomyces cerevisiae* to ascladiol, a less toxic compound that still maintains some toxicity. Conversely, *R. glutinis* LS11 degrades patulin to desoxypatulinic acid under aerobic conditions. Therefore, a possible practical application of this metabolization process by *R. glutinis* LS11 would not imply the costly removal of oxygen. The enzyme(s)/gene(s) responsible for toxin conversion could also be used for producing apples capable of degrading patulin, as has already been described for transgenic rice plants harbouring trichothecene and zearalenone-inactivating genes

(Higa et al., 2003) or possibly be used in an industrial process to degrade patulin from contaminated apple juice.

#### **16.4. HOW BIOCONTROL AGENTS CONTROL DISEASE AND THE ROLE OF MYCOTOXINS IN A WIDER PERSPECTIVE**

The major challenges for postharvest biological control based on antagonistic microorganisms are the economic feasibility of its implementation and the achievement of levels of efficacy and reliability that are comparable to those of chemical control. In this regard, the above-mentioned integrated approach, relying on both the biocontrol agents and reduced dosages of chemical fungicides, can actually contribute to achieving these goals. Nevertheless, more research efforts are needed in order to lower the effective biomass and the inherent production costs of antagonistic microorganisms to be used in practical applications, and to enhance the efficacy of these beneficial microbes. Suitable formulations of these agents could play a crucial role in their effectiveness by increasing their dispersion and colonization on fruit skin, by prolonging their survival under practical conditions, and by enhancing the mechanisms of action underlying their biological activity. New information on the ecological role of mycotoxins and the processes adopted by the mycotoxigenic fungi during infection of the fruit tissue could also help in a more successful utilization of biocontrol agents.

##### **16.4.1. MECHANISMS OF ACTION OF POSTHARVEST BIOCONTROL AGENTS**

Most studies on the mechanisms of action of biocontrol agents have been carried out on yeasts applied in fruit/postharvest pathogen systems involving pome and citrus fruits. These studies were aimed at identifying traits playing a major role in the antagonistic activity thus paving the way for their physiological and genetic enhancement.

While early reports indicated that competition for nutrients and the fast growth rate of antagonists played a major role in biocontrol activity (Droby et al., 1989), subsequent studies have suggested a much more complex interaction between the antagonist, pathogen and commodity (Droby and Chalutz., 1994). It was shown that yeast antagonists are able to form a biofilm, thus preventing germination cues and/or nutrients from reaching the pathogen, and that some of the yeast antagonists adhere to and parasitize pathogen hyphae (Wisniewski et al., 1991). The latter report was recognized as the first instance of the ability of a yeast to parasitize a higher fungus (Elad, 1995). The activity of biocontrol yeasts does not appear to rely on undesirable antibiotic production, whereas competition for space and nutrients is still considered to be

a major mechanism of many biocontrol agents effective on pome fruits (Castoria et al., 1997, 2001; Janisiewicz and Korsten, 2002) and citrus fruits (Bull et al., 1997; Poppe et al., 2003). Competition for nutrients negatively affects *P. expansum* because this fungus, like *B. cinerea*, is a wound-invading necrotrophic pathogen whose conidia require freely available nutrients for germination and subsequent infection of plant tissue. Successful competition for space and nutrients (as well as other biocontrol mechanisms) requires the timely colonization of wounds by the biocontrol agents (Janisiewicz and Korsten, 2002), i.e., the so-called “wound competence” of these microorganisms (Droby and Chalutz., 1994). Castoria et al. (2003) showed that a timely wound colonization appears to rely on the resistance of the biocontrol yeasts used in their study to the reactive oxygen species hydrogen peroxide ( $H_2O_2$ ) and superoxide anion ( $O_2^-$ ) produced by the apple tissue as a consequence of wounding.

Studies at a biochemical and genetic level also suggested a role in postharvest biocontrol of yeast extracellular enzymes ( $\beta$ -glucanases and chitinases) that depolymerize fungal cell walls (Janisiewicz and Korsten, 2002). However, the importance of  $\beta$ -glucanases has recently been questioned. Disruption of glucanase-encoding genes and/or their over-expression does not apparently affect yeast biocontrol activity (Droby, 2001; Yehuda et al., 2001; Segal et al., 2002; Grevesse et al., 2003; Yehuda et al., 2003), although at lower yeast cell concentration some reduction of biocontrol activity was recorded (Bar-Shimon et al., 2004). Only when two glucanase-encoding genes were both disrupted, was a decrease in postharvest biocontrol activity of the yeast *Pichia anomala* observed (Friel et al., 2007). Possible explanations for the results reported in these studies could be that at a higher yeast cell concentration the role of glucanases could be masked by other mechanisms of action, such as competition for nutrients (Friel et al., 2007). Another possibility is that there are glucanases yet to be uncovered that are specifically induced upon the encounter of a biocontrol yeast with a fungus and/or fruit. In order to reveal fungal-fruit-inducible genes, it is first necessary to disrupt other glucanase genes. This was the method employed to uncover the entire set of plant-inducible pectate lyase genes of the bacterial plant pathogen *Erwinia chrysanthemi* that were not expressed on artificial media containing pectate, but only in the plant (Kelemu and Collmer, 1993). In addition, the above-mentioned studies on glucanases focused on enzymes with an *exo* mode of action. Glucanases with an *endo* mode of action, if any, might play a more significant role in a putative aggressive mode of biocontrol yeasts towards fungal pathogens.

Induced resistance has also been claimed to be a mechanism of the activity of postharvest biocontrol agents (Droby and Chalutz, 1994). It has been described for apples and citrus fruits, and it involves the production of glucanases and chitinases, structural barriers such as wall appositions, phytoalexins, and an increase in ethylene and activity of the enzyme phenylalanine ammonia lyase (El Ghaouth et al., 1998; Droby et al., 2002a, 2002b; El Ghaouth et al., 2003). Pretreatment of artificial wounds of stored apples

with the yeast-like fungus *Aureobasidium pullulans* L47 significantly decreased the incidence of *B. cinerea* and *P. expansum* infections and caused the onset of  $\beta$ -1,3-glucanase and chitinase activities in the wounded tissue (Ippolito et al., 2000). In this case, it was not possible to establish whether or not these enzyme activities were of host origin. Conversely, El Ghaouth et al. (2003) showed that treatment of wounds on fresh Red Delicious apples with the biocontrol yeast *Candida saitoana* significantly limited the lesion development caused by *B. cinerea* inoculated in untreated and physically separated wounds on the same fruits. The reduction of lesion development was accompanied by an increase in  $\beta$ -1,3-glucanase and chitinase activities both in the yeast-treated and in the untreated, physically separated wounds.

Induced resistance studies suggest that a deeper understanding of the tritrophic interaction plant tissue-pathogen-biocontrol agent could also help in the enhancement of antagonistic activity and protection of the crop. *Trichoderma atroviride* harbouring multiple copies of a glucose oxidase-encoding gene from *A. niger* was able to produce  $H_2O_2$  following induction by fungal pathogens (Brunner et al., 2005). This new trait conferred on transgenic *T. atroviride* a higher hyperparasitic activity against fungal pathogens and the capability of inducing systemic disease resistance in plants.

An analogous approach based on heterologous (or homologous) genes could be used for conferring on postharvest biocontrol agents the ability to degrade mycotoxins in situ and/or to enhance their antagonistic activity. Wisniewski et al. (2003) showed that overexpression in yeast of a lytic peptide belonging to the defensin family of antimicrobial peptides could enhance biocontrol activity. In this research, a defensin gene was isolated from peach (*Prunus persica*) bark tissues and overexpressed in a *Pichia pastoris* recombinant protein expression system. Yeast lines expressing the peach defensin protein completely inhibited germination of *Penicillium expansum* conidia. The effect, however, was fungistatic rather than fungicidal, as germination proceeded normally once the conidia were removed from the medium containing the transgenic yeast.

## 16.4.2. PATHOGENICITY FACTORS OF POSTHARVEST PATHOGENS

A new avenue of biocontrol research might open up based on new information about the pathogenicity strategies of *B. cinerea* and *P. expansum*, since biocontrol agents could interfere with the pathogenicity factors of these necrotrophic wound pathogens. This information could pave the way for the development of new control tools, such as facilitating the interference of biocontrol agents with specific factors of the pathogens that are involved in the infection process.

Castoria et al. (2003) suggested that biocontrol yeasts that are resistant to oxidative stress could also protect the apple tissue by providing it with exogenous antioxidant activity counteracting reactive oxygen species, which are known to be produced/induced by *B. cinerea* (van Kan, 2006) and the mycotoxigenic *P. expansum* (Hadas et al., 2007). The biocontrol activity of

bacteria protecting *Arabidopsis thaliana* and grapevine from *B. cinerea* through the possible interference with its pathogenicity factor oxalic acid (van Kan, 2006) has recently been reported (Schoonbeek et al., 2007). Production of  $H_2O_2$  during the infection process by *P. expansum* on apples is a by-product of glucose oxidation by a fungal glucose oxidase leading to the production of gluconic acid. This compound causes acidification of the apple tissue, as does oxalic acid for *B. cinerea*, thus favouring the production and the activity of other pathogenicity key factors such as cell wall degrading enzymes (ten Have et al., 1998; Prusky and Yakoby, 2003; Prusky et al., 2004). In the light of these findings, the action of sodium bicarbonate that has been proposed as a tool to control postharvest disease with a direct effect on the fungal pathogen (Wisniewski et al., 1995; Droby et al., 1997; Karabulut et al., 2001; Droby et al., 2002b; Porat et al., 2002) could be re-considered. This salt increases pH, therefore it could also interfere with the infection process by counteracting the acidification of the host tissue that is caused by fungal pathogens.

Mycotoxins such as the trichotecene deoxynivalenol produced by *Fusarium* spp. that appear to be involved in the development of the disease symptoms on their host plants are synthesized at an early stage of the infection process, and are therefore considered as virulence factors (Desjardins and Hohn, 1997; Kang and Buchenauer, 2002; Mesterhazy 2002).

The possible role of patulin in the infection process of *P. expansum* is yet to be explored (Desjardins and Hohn, 1997). Isolates of this fungus with more aggressive growth appeared to be able to produce the greatest amounts of patulin (McCallum et al., 2002). Conversely, Paster et al. (1995) reported the absence of correlation between patulin accumulation and symptoms, expressed as lesion diameters, of *P. expansum* infection on apples. As mentioned in section 16.2.3, however, lesion diameters can be considered only as a semi-quantitative index of pathogen growth in the decaying fruit tissue. On the other hand, patulin is known to react with and deplete an important cell antioxidant such as glutathione in animal systems, and to cause generation of reactive oxygen species (Barhoumi and Burghardt, 1996; Fliege and Metzler, 2000; Rychlik et al., 2004). Furthermore, patulin is stabilized by an acidic environment, such as that created through the secretion of gluconic acid by *P. expansum* during the infection process on apple fruits (Hadas et al., 2007). Therefore, the possible role played by this mycotoxin in the plant–fungus interaction is a topic for further investigation, under the common theme of the oxidative attack of plant tissues by necrotrophic pathogens.

### 16.4.3. MYCOTOXINS AND INTERMICROBIAL COMPETITION

The mycotoxin-producing species of *Penicillium*, *Aspergillus* and *Alternaria* that cause postharvest decay of fruits usually infect tissues that are weakened, injured or already infected by primary pathogens (Moake et al., 2005; Zimmerli and Dick, 1996). These fungi do not predominate in the microflora during the growing season; their epiphytic growth on surfaces of fruits and

berries is therefore presumably suppressed by the presence of other, competing microorganisms and by unfavourable environmental and nutrient conditions. *Penicillium* species were not commonly isolated from apple flowers and immature fruits (Pennyhook and Newhook, 1981). Also at apple harvest, their numbers were low, only to increase rapidly during postharvest storage (Teixidó et al., 1998). *Alternaria* species have a colonization profile over time that differs from that of *Penicillium* spp. Populations of *Alternaria* were present from petal fall to harvest, but increased towards the end of the season (Dugan and Roberts, 1994). In general, yeasts and bacteria appear to be the predominant colonizers early in the season, and filamentous fungi tend to appear later in the season and during storage (Spurr, 1994). Microbial populations of diverse habitats change over time, and this also holds true for the epiphytic microflora on fruits (Spurr, 1994). The natural succession of epiphytic yeasts on grapes over the season includes species of *Rhodotorula*, *Cryptococcus* and *Candida*, and the yeast-like fungus *Aureobasidium* during ripening, to be followed by and include species of *Hanseniaspora* and *Metschnikowia* at ripening (Fleet, 2003). Many of these yeast genera harbour biocontrol agents. The composition of the mycoflora will also depend on the cultivar on which it resides and on the climate. *Penicillium expansum*, which is normally ubiquitous, was isolated from wine grapes in the Douro region, but not from wine grapes of the same cultivars in the Vinho Verde region (Abrunhosa et al., 2001).

Since some mycotoxins have antimicrobial activity (e.g., patulin), they could possibly also be directly involved in intermicrobial competition under the environmental conditions that favour growth of the mycotoxin producer. The role of mycotoxins in microbial ecology of the fruit surface and in fruit wounds is not clear, since research on and the definition of these compounds are usually viewed from the perspective of their toxicity to man and animals. But it is possible that mycotoxins may play a direct role in intermicrobial competition and survival, much as was speculated regarding the ecological role of the phytotoxins (microbial toxins that inhibit plants) that also have antimicrobial activity, such as phaseolotoxin, tabtoxin, rhizobitoxine, tropolone, syringomycin and syringotoxin (Durbin 1982; Demain 1995; Wright, 1997). Secondary metabolites were considered in the past to be waste products from primary metabolism. This view has changed, and for many years now, they have been believed to exist in their own right (Lattanzio et al., 2006), providing the producing organism with a competitive advantage. Their biosynthetic gene clusters have often moved horizontally across genomes of different microorganisms. Patulin is an example of a mycotoxin that has well-documented antimicrobial activity (Scott et al., 1972), to more than 70 different species of microorganisms (McKinley and Carlton, 1991).

Biocontrol yeasts and bacteria that are used to control mycotoxigenic fungi must be able to resist the antimicrobial activity of a mycotoxin such as patulin. Indeed, if a mycotoxin has antimicrobial activity and is being produced from the time that the mycotoxigenic fungus resides on the surfaces of leaves and fruit in the field until and during postharvest, then the biocontrol agent needs to overcome its toxicity in order to establish itself.

It is interesting to note that very little attention has been given to this topic, when considering that this trait should be a prerequisite for the very survival and success of the biocontrol agent. Representatives of certain phylogenetic groups are known to produce a wide array of antimicrobials, such as species of *Penicillium*. A large number of *Penicillium* strains of diverse species were isolated from mycelial mats of *Rhizoctonia solani* that were taken from the rhizosphere of tobacco. Many of the *Penicillium* species produced antifungal substances, some of which were characterized (Nicoletti et al., 2004). *P. expansum*, the causal agent of blue mould in fruits produces many additional secondary metabolites and mycotoxins in addition to patulin (Andersen et al., 2004). The antimicrobials could play a role in microbial competition on fruit surfaces. For example, in a study by Serra and co-workers (Serra et al., 2006) *Penicillium thomii* was usually not recovered from grape samples taken in Southern Portugal, from which *Penicillium brevicompactum* was isolated, and *P. brevicompactum* was not isolated from grape samples taken in Northern Portugal, in which *P. thomii* was present. The two penicillia have similar ecophysiological niches, i.e., growth at low water activity and at low temperatures. The growth of one species appears to exclude the other, possibly through antibiosis (Serra et al., 2006).

The production of mycotoxins is dependent on environmental cues. Patulin and ochratoxin A are produced to a different extent depending on the cultivar of apple and grape, respectively, that the *P. expansum* (Moake et al., 2005) or *A. carbonarius* (Zimmerli and Dick, 1996) strains have infected. The composition of the surrounding antagonistic microflora will thus be exposed to different mycotoxins depending on whether or not the environment is conducive to their biosynthesis, and will change accordingly.

## 16.5. FUTURE PERSPECTIVES

Contamination of fresh fruits with fruit pathogens capable of producing mycotoxins is regarded as one of the most important criteria for food safety. Tremendous efforts have been made worldwide to develop safe and effective strategies to control these pathogens.

The future of chemical fungicide control of postharvest mould-causing fungi is questioned and its use has been placed under scrutiny. This is because of (1) the failure to effectively control mould in several harvested fruit commodities due to fungicide resistance, (2) the concern of consumers about the possible effects of toxic residues on human health and the environment, and (3) the restrictions imposed by authorities on the use of agro-chemicals. These issues, which were the driving-force behind the development of reduced-chemical postharvest disease control measures, have now become an economic imperative, not just an option. The use of alternative non-chemical control methods as stand-alone treatments, however, does not provide the efficacy and consistency required under commercial postharvest situations.

As indicated, the use of the available postharvest biocontrol products has so far been rather limited given the potential market. While some of the reasons for



the lack of success have been addressed in many review articles, a large measure of their future success will depend on market conditions. Due to the reliable performance of synthetic fungicides, their long history and relative ease of use, continued consumer and regulatory demands on the elimination or restricted use of these fungicides must continue if they are to be replaced by biological products. Additionally, new biological postharvest products must be adaptable and effective as a stand-alone product without needing additional inputs if they are to be competitive with synthetic fungicides. Also, postharvest biologicals must begin to address problems of decay management in commodities where postharvest disease is harder to control, such as stone fruits and berries. Lastly, the huge potential of providing extended decay control to the consumer, prior to and after commodity purchase, through the use of antimicrobials in modified and intelligent packaging should be recognized. The greatest hope for a biological approach (using a broad definition of biological control) lies in a further understanding of the mechanism(s) of action of microbial antagonists and natural products, innate and induced resistance in the host, and the biology of decay pathogens. It is expected that this knowledge will lead to new, innovative approaches for controlling decay in harvested commodities and presents the best hope for the future of the biological control of postharvest disease.

It is an enormous challenge to meet the demands for improved quality and safety, and reduction of postharvest losses due to mould infections in an affordable and environmentally compatible manner. After decades of research, several questions regarding the relationship between infection levels occurring in the field and the development of postharvest decay remain unanswered. In this regard, preharvest application of postharvest biocontrol agents has been suggested (Ippolito and Nigro, 2000). To develop more efficient methods for controlling postharvest mould contamination, it is essential to elucidate the relationship between the infection of various plant parts in the field and the incidence of disease or toxic contaminants. The development of control strategies based on a holistic approach in which modelling and prediction systems, early detection techniques, biological and physical methods and cultural practices should be adopted.

## 16.6. CONCLUDING REMARKS

Mycotoxins are produced in fruits during postharvest through secondary metabolism by rot-causing fungal species belonging to the genera *Penicillium*, *Aspergillus* and *Alternaria*. Biological control of these pathogens can be an alternative and complement to existing methods of control. It holds particular promise in the postharvest environment since it can be carefully regulated, and the application of biocontrol agents can be integrated into existing agricultural practices and handling pre- and postharvest. A few commercial biocontrol products for postharvest rots are commercially available, but so far their efficacy when applied alone is not yet completely satisfactory from the farmer's perspective. Certain biocontrol agents have more than one beneficial effect.



For instance, the biocontrol yeasts that are able to protect fruits and grapes from infection and rot by mycotoxigenic fungi are also able to reduce the accumulation of mycotoxins. The ability of yeasts to degrade patulin and ochratoxin A are features that can be exploited in alternative industrial applications. In order to develop biocontrol further for practical use, it is imperative that we understand the modes by which the beneficial microorganisms affect biocontrol, the role of mycotoxins in plant disease and the impact that mycotoxins might have on the growth of epiphytic microflora and biocontrol agents. The future of practical postharvest biocontrol will depend largely on the development of methods that prove consistent in their performance, and that preferably cover several minor crops in addition to commodities where postharvest diseases are hard to control. The market situation, including the aptitude of consumers and industry to embrace this new technology, will greatly influence the transition to implementing environmentally friendly methods in the battle against mycotoxigenic fungi.

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# Effect of Processing on the Mycotoxin Content in Fruit Juice

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## ABSTRACT

Contamination of apple juice by patulin has attracted considerable attention due to the ubiquitous consumption, economic importance and international trade in apple juice concentrates. Numerous studies have highlighted the need for processing apples of high quality, and patulin levels in the final juice product have been suggested as an indicator of the original fruit quality. Storage of harvested apples under adverse conditions such as on open deck can rapidly lead to fruit decay and enhanced patulin levels. Limiting the entry into the processing facility of damaged and decayed apples by manual sorting and trimming or the use of mechanical means such as high pressure sprays is the first essential step to control patulin levels in the final product. Once present in the juice being processed, the most effective control measure is the use of activated charcoal, of which several commercial products are available and have been tested for efficacy. Subsequent to the production of juice concentrate, processes such as fermentation or the addition of additives such as ascorbic acid can reduce patulin levels. The prevention of contamination problems associated with other mycotoxins in fruit juices, such as ochratoxin A in grape juice and its subsequent appearance in wine, also requires the use of high-quality fruit, minimizing conditions conducive for fungal growth and toxin production in the process or the use of suitable adsorbents or fining agents.

## 17.1. INTRODUCTION

Although a large number of mycotoxins are known to be produced by toxigenic fungi, especially under suitable culture conditions, only relatively few occur naturally in fruits and are considered to have economic or health

importance. In this regard, the mycotoxins that have elicited the most attention on whole fruit are aflatoxins in figs, patulin in apples and berries, and *Alternaria* toxins such as alternariol, alternariol methyl ether and tenuazonic acid in tomatoes and apples. Most of these toxins have been detected in commercial fruit juices (Drusch and Ragab, 2003). The natural occurrence of mycotoxins in fruit juices can conceivably arise from fungal contamination and consequent toxin production in the fruit prior to or during harvest, during storage prior to factory processing or during the processing itself. The key to the production of contaminant-free juice lies ideally in the use of sound uncontaminated fruit, a situation that is possible to achieve in small home industries, but not always on a large industrial scale.

Of the various fruits subjected to industrial juicing, apples have been almost exclusively the subject of intensive investigation, partly due to the high prevalence of patulin in apple juice worldwide and partly due to apple juice concentrate being widely traded internationally both as a source of apple drinks and as a base for other mixed juices or beverages.

The presence of patulin in apple juice has been used as a marker for the quality of fruit used in processing, as well as a parameter for setting the trading price (Doores, 1983). Internationally, many countries have set maximum tolerated levels (MTLs) for patulin in apple juice. During the recent FAO survey of worldwide regulations for mycotoxins conducted during 2003, 48 countries reported the presence of MTLs for patulin with 44 countries having set the limit at the level of 50 µg/l (ppb) (FAO, 2004). The European Union, while setting a limit of 50 ppb for fruit juices, has specifically lowered it to 25 ppb for solid apple products such as compotes and purees, and to 10 ppb for apple products intended for baby food and infant formulae (FAO, 2004). The presence of legislated limits and their enforcement in international trade has brought greater awareness of the problems of patulin contamination to the apple-processing industry and has resulted in greater emphasis being placed on processes that lessen the contamination of the final juice product.

Although less is known about its natural occurrence and the source of the toxin, grape juices, particularly red grape juice, have in some instances been found to contain low levels of ochratoxin A (Zimmerli and Dick, 1996; Drusch and Ragab, 2003). The source of ochratoxin A in grape juice is assumed to be similar to that of ochratoxin A in wine and much recent research has been focussed on this question (Cabañes et al., 2002; Serra et al., 2003; Battilani et al., 2003; Kozakiewicz et al., 2004). Although the consumption of wine is widespread and the industry is of considerable economic importance, the fate of ochratoxin A during wine making has only recently been studied. Even less is known about the situation as regards the effects of processing on ochratoxin A levels in grape juice. For the reasons discussed above, this chapter will mostly review the factors affecting patulin levels in apple juice during industrial processing and will only make a short review on the situation regarding ochratoxin A in grape juice.

## 17.2. PATULIN IN APPLE JUICE

### 17.2.1. BACKGROUND

The fungus *Penicillium expansum* Link, a well-known pathogen of apples and pears that occurs in soil, is commonly associated with the “blue mould rot” of apples and is the main organism responsible for the production of patulin in apples (Doores, 1983). Although patulin as a fungal metabolite was originally discovered and tested as an antibiotic and possible cure for the common cold during the 1940s (Clarke, 2006), it was only later detected as a natural contaminant of apple juice (Scott and Somers, 1968; Scott et al., 1972; Stott and Bullerman, 1975). International concern over its natural occurrence was enhanced following a survey conducted in 1992 by the Ministry of Agriculture, Fisheries and Food (MAFF), which showed levels in consumer products as high as 434 µg/kg (MAFF, 1993).

Patulin is regarded as an *in vivo* mutagen by the United Kingdom Committee of Toxicity, Mutagenicity and Carcinogenicity of Chemicals in Food, Consumer Products and the Environment (1992). It also has teratogenic properties, and shows immunotoxic, neurotoxic and gastrointestinal effects in rodents (Hopkins, 1993). Consequently, international attention has become more focussed on the occurrence and control of this food contaminant. The British Soft Drinks Association (BSDA) published a code of practice aimed at the reduction of patulin levels (BSDA, 1993), whereas more recently the Codex Committee on Food Additives and Contaminants passed “Code of Practice for the Prevention and Reduction of Patulin Contamination in Apple Juice and Apple Juice Ingredients in other Beverages” (Codex Alimentarius Commission, 2003). These codes of practice deal with a wide range of aspects, including recommendations for good agricultural practice (GAP) at the preharvest stage, the harvesting and transport of fruit, post-harvest handling, storage and finally good manufacturing practices (GMP). These codes identified the importance of GAP, since the key to low levels of patulin contamination in the final product lies in the use of good quality fruit with a minimum fungal contamination and decay. Studies in which apple juice has been made from tree-picked apples and from ground-harvested (dropped) apples both fresh and stored has indicated the extent to which final patulin levels can depend on apple quality (Jackson et al., 2003). However, it should be recognized that in many instances, factories have limited control over the quality of fruit delivered for processing and hence industrial measures to control and reduce patulin contamination are essential.

The intent of this section is to address the effects of industrial processing on patulin levels and consequently will cover issues including post-harvest factory storage, washing and sorting of fruit, processing of juice concentrate, further special treatments such as fermentation and the stability of patulin in juice.

### 17.2.2. POST-HARVEST STORAGE

Post-harvest storage refers to the period between apple harvest and the fruit entering industrial processing. Ideally, fruit should be processed as soon after harvest as possible. However, given limited processing capacity, fruit received by the factory during peak harvesting time is generally stored for later processing and thus measures to reduce fungal growth and delay post-harvest decay during storage are frequently applied. This is optimally achieved at low temperatures ( $<4^{\circ}\text{C}$ ) and under modified atmospheres, which typically contain 3 percent carbon dioxide and 2 percent oxygen (Paster et al., 1995). However, even under these conditions, *P. expansum* can grow and produce patulin to a limited extent depending on the fungal strain and on the apple cultivar involved (Lovett et al., 1975a). Studies have highlighted these interactions between apple cultivar, storage temperature, fungal strain and modified atmosphere composition and also the potential for suppression of patulin formation but not of fungal pathogenicity (Morales et al., 2007; Vismer et al., 1996; Paster et al., 1995). Although organic vapours such as trans-2-hexenal have been tested as fungal control agents, the production of off-flavours in some apple cultivars can restrict their application (Neri et al., 2006a). Trans-2-hexenal has similarly been shown to be effective against *P. expansum* and patulin production in Conference pears, although off-odours and off-flavours are also a concern (Neri et al., 2006b).

The lack of sufficient cold storage and modified atmosphere facilities and restricted processing capacity may lead to fruit being kept in open deck storage for variable time periods. Under these sub-optimal conditions, exposure of apples to the prevailing weather conditions is detrimental to fruit quality and may lead to enhanced fungal growth and toxin contamination. A study was conducted in which Granny Smith apples (150 tons in total) were stored in industrial bins in the normal factory deck area for periods of 7, 15 and 33 days (Sydenham et al., 1997). Representative samples for analysis were drawn during the processing of the 50 tons assigned to each time point. Over the time of the experiment, *P. expansum* counts rose from  $3.21 \log_{10}$  colony forming units (cfu)/g at 7 days to  $4.93 \log_{10}$  cfu/g at 15 days storage and  $5.20 \log_{10}$  cfu/g after 33 days storage. Patulin levels rose from  $90 \mu\text{g/kg}$  at 7 days to  $395 \mu\text{g/kg}$  and  $2445 \mu\text{g/kg}$  after 15 and 33 days, respectively. Although the exact fungal incidence and toxin levels reached during storage in this study depended on extraneous uncontrollable variables such as the initial presence of fungal spores and the weather conditions over the storage period, the results do indicate the extreme problems faced by factory processors if apples are retained in deck storage for extended periods of time.

A portable drencher, capable of treating apples in single storage bins, has been described and used for studies on the possible control of fungal decay by drenching with solutions of sodium bicarbonate (2 percent), the yeast *Metschnikowia pulcherrima* or a combination of both (Janisiewicz et al., 2005). After drenching, the fruit bins were placed in regular cold storage ( $2^{\circ}\text{C}$ ) and the

fruit decay (but not patulin levels) was assessed after 3 months. The combination treatment was found to significantly reduce the incidence of fungal decay. The legal and social constraints on the use of synthetic fungicides during fruit storage has led some researchers to investigate the use of natural food ingredients as possible fungicides during apple storage. Substances showing potential to control *P. expansum* and/or reduce patulin levels include lemon and orange oil (Hasan, 2001) and hydrogen peroxide (Venturini et al., 2002). Other workers have suggested the use of gamma-radiation, which at doses of 1.5 and 3.5 kGy on apples, was shown to reduce total fungal counts and patulin levels relative to controls after storage below 10°C for 28 days (Aziz and Moussa, 2002).

### 17.2.3. INITIAL PROCESSING

Considering the constraints on harvesting and storage already discussed, it is inevitable that some decayed apples will enter the processing facility when individual bin consignments of the order 360 kg are unloaded into the process stream. Typically, apples are simultaneously washed to remove dirt and any chemical residue and transported by flume water into the factory. This stage, which can incorporate a hand-sorting table, presents the only opportunity to visually inspect and sort the apples prior to juicing. As in other mycotoxin contamination problems, patulin is not homogeneously distributed in the incoming apples, but is concentrated in the fungally infected rotten apples. Laboratory studies using individual apples from three different varieties inoculated with three different *P. expansum* strains and allowed to incubate for 14 days showed that careful laboratory trimming of the rotten tissue could remove over 93 percent of the toxin from the apple (Lovett et al., 1975b). Further laboratory studies on the diffusion of patulin from the rotten tissue into sections of the apple without visible rot showed that patulin can diffuse into whole apple tissue, but that the levels of patulin rapidly decrease to negligible levels over a distance of 1 cm from the rot (Taniwaki et al., 1992; Rychlik and Schieberle, 2001; Marin et al., 2006). These laboratory studies point to the possibility of effectively using manual sorting and trimming to remove significant amounts of patulin prior to its entry into the factory juice stream.

The actual level of patulin removal that could be achieved during a commercial operation using flume water wash, removal of wholly rotten apples and trimming of those with less severe decay was studied on three occasions, each during a separate commercial operating season (Sydenham et al., 1995, 1997; Leggott et al., 2000). In an initial study using over-ripe fruit, apple samples were collected during normal factory operation from the storage bins being processed, from the flume after a normal wash operation, from the end of a sorting table after removal or trimming of decayed apples, from the bin containing rotten material removed from the process and from the flume water itself (Sydenham et al., 1995). Results showed that the mean level of patulin in apples entering the process from the bins was 920 ppb, falling to a mean level

of 190 ppb after the normal wash operation and to 55 ppb at the end of the sorting table once visual inspection and removal of damaged fruit or trimming of decay had occurred. The effectiveness of this process was also demonstrated by the mean level of 2335 ppb measured in the material removed at the sorting table and by the level of 1960 ppb measured in flume water 2 hr after its replacement with fresh water. A subsequent study involved the lots of stored (for 7, 15 and 33 days) Granny Smith apples described in the preceding section (Sydenham et al., 1997). Samples withdrawn at the same stages of the process as described above showed a similar trend of decreasing mean patulin levels. The results of the study using apples stored for 33 days on open deck were mean levels of 2445 ppb in the bin, 695 ppb after washing, 405 ppb after sorting, with a collected rotten fraction of 6235 ppb. These results illustrate that although washing, sorting and trimming can significantly reduce levels of patulin entering the later stages of processing, they cannot alone achieve adequate removal of patulin on an industrial scale if the quality of fruit deteriorates.

Besides tumbling in flume water, the washing of apples prior to juicing can also be achieved by high-pressure water jets, sometimes at several points, which have the secondary benefit of helping to remove rotten tissue. A study on the effects of processing on patulin levels in commercial apple juice showed losses of patulin of up to 54 percent during the use of these jets, up from a maximum of 31 percent achieved in the conventional wash process (Acar et al., 1998).

#### 17.2.4. PROCESSING

Just as the initial processing of apples, which involves washing and handling, can be achieved industrially in different ways, so the basic factory operations of juicing and pressing, pasteurization, depectinization, filtration, clarification and concentration required to produce apple juice concentrate from washed apples can be achieved in a variety of ways with different commercial processing equipment. A number of studies have addressed all or some of these processes on either a laboratory or an industrial scale (Acar et al., 1998; Leggott et al., 2000; Bissessur et al., 2001; Gökmen et al., 2001). Laboratory separation of apple juice from the homogenized pulp showed that around 50 percent of the original patulin was retained in the solid material, probably due to binding of the toxin (Bissessur et al., 2001). Similarly, binding of patulin during the analytical determination of patulin in cloudy apple juice has been demonstrated and it was surmised that protein in the suspended solids of the cloudy juice was responsible (Baert et al., 2007). However, factory-scale investigations on the entire processing line from apples to juice concentrate failed to detect such a decrease during the first pressing of the mash from the milling stage (Acar et al., 1998). A small decrease in levels at the second pressing was observed, but could be related to the dilution effect of the additional water added to the pressing waste from the first stage. This industrial scale study went on to determine patulin losses across the processes

of aroma recovery, depectinization, clarification, filtration and evaporation to produce apple juice concentrate. The filtration step was performed either on a precoated rotary vacuum filter or by ultrafiltration (Acar et al., 1998). The most significant decrease in patulin levels during these trials occurred during clarification with gelatine and bentonite and rotary vacuum filtration, when a loss of 35 percent was measured. Further studies were performed by these authors on a laboratory scale to determine the efficiency for patulin removal of various clarification treatments. In all, six different treatment regimens were investigated, namely flocculation with gelatin and bentonite with and without activated charcoal followed by kieselguhr filtration; ultrafiltration with and without preflocculation with gelatin and bentonite; and ultrafiltration with subsequent adsorbent resin (polystyrene-DVB-based macro porous) or polyvinylpyrrolidone (PVPP) treatments (Gökmen et al., 2001). Adsorbent resins are used during apple processing for the removal of yellow-brown colour and phenolic compounds. Patulin removal was lowest during ultrafiltration alone (2.6 percent) and rose to 11.0 percent during ultrafiltration with subsequent adsorbent polystyrene-DVB resin treatment. The most significant reduction in patulin levels was achieved in the activated charcoal treatment, which showed a reduction of 40.9 percent.

In another study, conducted during normal factory operation, the effect on patulin and *P. expansum* counts of four sequential stages during manufacture of juice concentrate was investigated (Leggott et al., 2000). The stages were preconcentration, depectinization/activated charcoal/ultrafiltration, passage through a resin-based absorber and the final concentration. The charcoal was added to the depectinization tank after the filling of the tank prior to ultrafiltration. A significant increase in patulin concentration from 105 ng/ml to 165 ng/ml was observed across the pre-concentration stage. As the results were not adjusted for the increase in Brix level during this process, the increased patulin level was largely due to the increase in the concentration of the juice. In addition, increasing the temperature during pasteurization significantly reduced the mean fungal counts from 4.07 to 0.79 log<sub>10</sub> cfu/ml. It was postulated that this process may fragment the fungal spores and hyphae present in the juice, which releases patulin and possibly contributes to the observed increase in patulin contamination. Of the remaining process operations investigated, only the depectinization/activated charcoal/ultrafiltration step achieved a significant reduction in patulin with levels decreasing from a mean  $110 \pm 35 \mu\text{g/l}$  measured in samples withdrawn during the filling of the 80 000 L depectinization tank to  $75 \pm 18 \mu\text{g/l}$  measured in the corresponding juice exiting the ultrafiltration unit. The decrease was ascribed to the action of the activated charcoal (Leggott et al., 2000).

A side product of the apple juice concentration process is apple aroma, which is a distillate consisting mostly of water with ppm levels of various aroma compounds. The product can be added back to juice to restore the natural flavour or used in other food applications as a natural flavouring agent. Based on theoretical considerations of its polarity and volatility, patulin would not be expected to be carried into this product. This assumption has been



tested on a laboratory scale as well as by the analysis of several commercial aroma samples (Kryger, 2001). Results confirmed that any carry over is negligible.

### 17.2.5. POST-PRODUCTION OPERATIONS

Depending on the quality of apples being processed, patulin levels in the final apple juice concentrate product may still be above the maximum tolerated level required by the customer. The use of activated charcoal, either during the original process or in later reprocessing, represents the clearest mechanism whereby patulin levels can be reduced to meet these criteria. Sands et al. (1976) showed that patulin could be removed from apple juice by the application of activated charcoal (NORIT 211) at a level of 5 mg/ml, which pointed to the possible use of charcoal on an industrial scale. A carbon-based composite material has been developed in which activated carbon was bound to granular quartz to produce a fixed-bed adsorption column with good bed porosity and its effectiveness in patulin removal was demonstrated (Huebner et al., 2000). Interest by commercial suppliers of activated carbon to the apple industry has led to a systematic assessment of various carbon types (Leggott et al., 2001). Activated carbons are defined as highly porous carbonaceous materials, which provide large surface areas with adsorptive properties. The two main activation processes are referred to as “steam activation” and “chemical activation” and produce different surface properties and different pore shapes, sizes and distributions. These pores are differentiated into three categories, namely micro-pores (radius below 1 nm), meso-pores (radius 1–25 nm) and macro-pores (radius larger than 25 nm). The actual adsorption takes place in the micro- and meso-pores. In powdered carbon, access to these is not a problem due to the small particle size, whereas in granular carbons the macro-pores are required to provide ease of access to the adsorption sites (NORIT Nederland BV, 1995). Laboratory studies on naturally contaminated apple juice showed steam-activated carbons (NORIT SA4 and SX4) to be superior to a chemically activated NORIT CA1 at dosages of 1 g/l in 12°Brix juice at 55°C (Leggott et al., 2001). This difference was ascribed to the relative proportions of meso- and micro-pores in steam versus chemically activated carbon. Patulin removal from juices of higher solids concentration (20°Brix) required higher levels of carbon to achieve similar removal efficiency, whereas processing temperatures between 30 and 65°C did not influence carbon efficiency for the most efficient carbon tested (NORIT SA4).

The reaction of patulin with a number of naturally occurring or added juice components has been extensively documented. Patulin reacts readily with compounds such as cysteine, thioglycolic acid and glutathione, which contain sulfhydryl groups (Riley and Showker, 1991). Indeed, the use of SH-containing reducing agents such as cysteine has been mooted as a possible additive to cattle feed to reduce its toxicity in ruminants consuming contaminated hay or silage (Morgavi et al., 2003). Another well-documented additive that reduces patulin

levels is ascorbic acid (Brackett and Marth, 1979; Steiner et al., 1999). Recent work using an aqueous model system has suggested that patulin is decomposed by free radicals generated by the oxidation of ascorbic acid and that the reaction is catalysed by free metal ions (Drusch et al., 2007). The requirement for oxygen in this reaction and the low oxygen content in the headspace of food packages suggest that addition of ascorbic acid prior to filling would not be effective as a decontamination strategy. Other compounds which have been shown to reduce patulin levels in juice include the B vitamins, thiamine, pyridoxine and calcium pantothenate (Yazici and Velioglu, 2002) and sulphur dioxide, which may be used as a juice preservative (Steiner et al., 1999). Effectiveness of all these additives depends on concentration, time and type of apple juice.

It has been known for over 30 years that fermentation of apple juice to produce alcoholic cider causes a reduction in patulin levels. Patulin is a broad spectrum antibiotic, but only partially inhibits the growth of *Saccharomyces cerevisiae* at levels up to 200 mg/l (Moss and Long, 2002). Harwig et al. (1973) showed the disappearance over 2 weeks of patulin in apple juice fermented with *Saccharomyces cerevisiae* and *Saccharomyces ellipsoides*. Stinson et al. (1978) studied the removal of patulin at initial levels of 15 mg/l in apple juice using eight commercial yeast strains in three typical American alcoholic fermentation procedures. In all cases, 99–100 percent of the patulin disappeared over the fermentation period of 10–14 days at room temperature (23–25°C). The chemical processes responsible for the disappearance of patulin are complex and not fully understood, although recent studies using <sup>14</sup>C-labelled patulin have shown the presence of two major metabolites, one of which was identified as a reduction product, E-ascladiol, which is presumed to be further degraded during fermentation (Moss and Long, 2002). Although patulin is removed during fermentation, commercial ciders have been found to contain patulin (Stinson et al., 1978; Leggott and Shephard, 2001). The reasons for this could lie either in the use of extremely contaminated fruit juice or in the use of alcohol-intolerant yeast strains that cause arrested fermentations or the practice of adding unfermented juice to adjust the sugar content and flavour of the cider after fermentation.

### 17.2.6. STABILITY OF PATULIN DURING JUICE STORAGE

Patulin is a heat-stable compound in aqueous acid solutions (pH 3.5–5.5) (Lovett and Peeler, 1973). Depending on temperature and storage time, it has been shown to have considerable stability in apple juice. Pohland and Allen (1970) found no degradation in commercial apple juice stored (at unspecified temperature) for 10 days, and Wheeler et al. (1987) stored non-alcoholic apple cider for 1 month at 22°C without detectable loss of patulin. However, Scott and Somers (1968) reported a mean 11 percent reduction in patulin in canned apple juice over 3 weeks storage at 22°C, rising to a mean 49 percent reduction after 5 weeks, whereas patulin was stable when stored in fresh apple juice for 3 weeks at 22°C. During experiments on the reduction in patulin levels by

fermentation, Stinson et al. (1978) showed a 10 percent reduction in patulin in their controls kept at room temperature (23–25°C) for 2 weeks. Apple juice concentrates obtained from a commercial source and stored at 22 and 4°C showed reductions of 42.1 and 5.1 percent over a 1-month period, whereas 100 and 35.8 percent reductions were observed over a 6-month period, respectively (Yazici and Velioglu, 2002). However, storage of apple juice concentrate over these long periods at 22°C also leads to significant deterioration of juice quality parameters such as clarity, colour and turbidity. The extent to which patulin loss is due to reaction with naturally present ascorbic acid or with compounds containing sulphydryl groups is unknown, but could account for the variability in results of storage experiments.

### 17.3. OCHRATOXIN A IN GRAPE JUICE

The first report of ochratoxin A in grape juice and in red and white wine was by Zimmerli and Dick (1995, 1996). The source of this contamination has been the subject of some debate. Recent mycological studies on grapes in France (Bejaoui et al., 2006), Portugal (Serra et al., 2003), Spain (Cabañes et al., 2002; Belli et al., 2006), Italy (Battilani et al., 2003, 2006a), Israel (Guzev et al., 2006), Greece (Tjamos et al., 2006) and other countries in the Mediterranean basin (Kozakiewicz et al., 2004) have shown the overwhelming presence of black *Aspergillus* species, with 90 percent or more of the isolates falling into the section *Nigri*. Significantly, fungi of this section were also isolated from asymptomatic berries. Across the Mediterranean region, it was reported that of the isolates in this section, 30 percent were identified as *A. carbonarius*, most of which were high producers of ochratoxin A (Kozakiewicz et al., 2004). Studies on black *Aspergillus* species isolated from grapes collected throughout Italy showed OTA production by strains of *A. carbonarius* (22 of 23 strains), *A. tubingensis* (5 of 20 strains) and *A. niger* (3 of 15 strains) (Perrone et al., 2006). *A. carbonarius* is predominantly responsible for OTA contamination of grapes in Europe (Battilani et al., 2006b). It has been isolated from grapes and also been identified as a major producer of OTA in South America (Chulze et al., 2006) and Australia (Leong et al., 2006a). The presence and levels of ochratoxin A in grapes in the field depend on climatic and weather factors (Battilani et al., 2003, 2006a, c; Kozakiewicz et al., 2004). In addition, ochratoxin A levels can greatly and randomly vary among bunches and plants within a vineyard. For this reason, any assessment of ochratoxin A contamination in the field requires systematic field sampling for acceptable results (Battilani et al., 2006d).

As the major commercial products of the vineyards are table grapes, dried vine fruits and wines, rather than grape juice, more attention has focussed on the potential problems of mycotoxins in wine and dried fruit than in commercial grape juice. Health effects associated with OTA include renal disease (nephrotoxicity and nephrocarcinogenicity), immunological effects as well as embryotoxicity and teratogenicity (Benford et al., 2001). Little information is

available on the effects of processing of grape juice on the ochratoxin A levels in the final product. As in other juice products, sound fruit is the first prerequisite for non-contaminated juice. Studies on the fate of OTA during vinification have indicated that wine processing does not destroy OTA, but that the toxin is removed by binding to grape solids and precipitated proteins (Grazioli et al., 2006; Leong et al., 2006b). The levels of OTA in the liquid phase are greatly reduced at each processing stage that involves a solid-liquid separation, the greatest reduction occurring at pressing. In small-scale trials using white Semillon grapes inoculated on the vine, the laboratory simulation of pressing the crushed grapes (must) by hydraulic press and the clarification of the cloudy juice by sedimentation at 1°C for 4 days resulted in a 97 percent removal of the total OTA present in the must (Leong et al., 2006b).

Similar to the situation with wines, red juice contained higher ochratoxin A levels than white (Majerus et al., 2000). The reasons for this are not agreed, but may arise from mould growth after harvesting of the grapes and during long-time enzymatic treatment of the crude juice at elevated temperature used to improve the colour of the juice and/or wine. Majerus et al. (2000) have suggested that this enzymatic treatment of crude juices at elevated temperatures can lead to enhanced ochratoxin A levels in red wines. In the wine industry, the use of fining agents for clarification has been shown to reduce ochratoxin A levels (Castellari et al., 2001). The use of these agents in an additional clarification step in white grape juice may also account for the lower levels of OTA contamination found in white wines (Leong et al., 2006a,b). Laboratory studies on a range of inorganic and organic products showed the potential of fining agents such as potassium caseinate, gelatin, egg albumin and activated carbon to remove ochratoxin A in wine by adsorption. The most effective agents were the activated carbons, which removed up to 60 percent of the initial toxin. However, charcoal-containing agents can also influence the final colour of the product. Corresponding measures in the grape juice industry would similarly be expected to reduce the levels of ochratoxin A in the final product.

## 17.4. CONCLUSION

Patulin in apple juice is considered the most widespread contaminant problem faced by the fruit juice industry, with other toxins, such as ochratoxin A in grape juice, occurring sporadically at relatively low levels. The presence of fungal contamination, especially *P. expansum*, and the consequent patulin contamination of apple fruit entering the industrial process is difficult to entirely avoid. Nevertheless, suitable factory operating procedures and, when necessary, additional processing with charcoal can largely eliminate this toxin from the final product. With adequate analytical monitoring, factories should be capable of consistently achieving a final juice quality that has patulin levels below the legislated limits set in many countries.

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# Means to Prevent Contamination with Patulin in Apple-Derived Produce and with Ochratoxin A in Wines

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## ABSTRACT

Patulin is produced mainly by *Penicilium expansum*, a common pathogen of apples and pears. The preferred way to avoid patulin contamination in the products is by trimming away rotten fruits or infected areas. However, since patulin may diffuse from rotten parts to healthy tissues, removing rotten areas can markedly reduce, but not totally prevent, patulin contamination in the product. Washing and handling is efficient in reducing the amounts of patulin that might be transferred from the produce to the product. Patulin is stable against acidic pH, and is relatively stable at high temperature. Significant reduction of the toxin can be achieved during fermentation, and the rate of disappearance is dependent, among other factors, on the yeast strain and the medium.

Removal of patulin can be achieved by binding to materials used during processing, e.g., charcoal and composite carbon. Additives added to apple products may reduce their patulin content, but in some cases the quality of the product was adversely affected. Patulin destruction by additives in food products is dependent, among other factors, on temperature and length of storage.

The amount of the toxin can be markedly reduced by the inclusion in the processing of simple and feasible means, applied separately or, preferably, in a combined treatment, thus ensuring that safe products reach the consumer.

Ochratoxin A (OTA) is present mainly in wines produced from grapes grown in hot and humid countries; and *Aspergillus carbonarius* is the main OTA

producer in those areas. It was evident from studies undertaken in Europe and in Mediterranean countries that red wines contain greater amounts of OTA than white ones. OTA producers are present in undamaged grapes and OTA may occur in symptom-free grapes, although in smaller amounts than in damaged fruits. Minimizing injuries caused in the field may significantly reduce the amount of OTA in the grapes. The period between early veraison and harvesting can be regarded as the critical period for OTA accumulation, which is related to the composition of the grapes. However, the level of the toxin may differ between years even within a given vineyard, as a result of differing climatic conditions. Differences in the aggressiveness of fungal strains and in the sensitivity of grape varieties may also affect the OTA level. Trimming away poor quality (shriveled, damaged or infested) berries may reduce the risk of OTA contamination. Several bacteria, fungi or commercial enzymes are able to degrade or to adsorb OTA. Consequently, the major efforts are focused on biological degradation of OTA, since this strategy is more promising than chemical or physical strategies. The OTA content is reduced during the vinification processes. The toxin is bound to the solid parts of the grapes and to the yeast cells used during the fermentation, and removal of these solids may reduce the risk of OTA contamination. Fining agents may also contribute to OTA removal, but their efficacy depends upon the type of wine produced. It is important to exclude the possibility that wine constituents that contribute to the organoleptic properties of the wine are not removed by these agents. Since significant amounts of OTA are eliminated during vinification, more studies aimed at better understanding of the mechanisms involved in OTA reduction through adsorption will lead to improved OTA removal, thus contributing to the achievement of OTA-free products.

## 18.1. INTRODUCTION

Mycotoxins, highly toxic fungal metabolites, elicit a range of diseases when consumed by humans and animals. These include hepatocellular carcinoma, damage to kidneys and other organs, hormonal disruption, immune suppression, and edema of the body cavity. The stability of the mycotoxins under food processing and other means of detoxification, along with their potential use in agro-terrorism, has placed them in the forefront of public health concerns. Numerous efforts have been exerted in seeking means for the elimination of mycotoxins from agricultural produce and/or processed foods, or for their detoxification. Ideally, decontamination procedures should meet the following main criteria: complete inactivation, destruction or removal of the toxin; no production or deposition of toxic residues in the food/feed; preservation of the nutritive value of the food; no alteration of the acceptability or the technological properties of the product; integration into the regular food-processing and preparation steps; cost effectiveness; ease of use; freedom from health hazards to workers; no danger of damaging or destroying food-processing equipment; and approval by regulatory agencies. To meet these criteria, removal of mycotoxin contaminants by physical means is an acceptable approach, primarily since the nutritional or technological properties of the produce will not change. However, the possibility of sorting produce to

remove mycotoxin-contaminated material is limited, and large-scale application is not always feasible. Thus, the best approach appears to be the destruction of mycotoxins in the raw materials. However, here again there are several obstacles: most mycotoxins are highly stable at high temperatures, and of the means tested for their elimination, only ammoniation has been confirmed to be an effective procedure. In addition, although treatments have been found that reduce levels of specific mycotoxins, no single method has been found equally effective against a wide variety of mycotoxins that may occur together. Thus, there is a great need for more strategies for mycotoxin detoxification.

Most of the studies of strategies for the management of mycotoxins in agricultural produce and food have dealt with fresh or dry grains, and nuts and their products. This can be attributed to the fact that grains are susceptible to many more mycotoxigenic species than fruits and vegetables, therefore, many mycotoxins appear in grains that do not appear in fresh fruits or vegetables. Moreover, most of the mycotoxins that can be found in fresh produce (e.g., patulin, citrinin, ochratoxin, alternaria toxins) are also produced on grains, but there are many mycotoxins which commonly appear on grains but not in fresh vegetable or fruits (e.g., *Fusarium* toxins).

## 18.2. APPROACHES FOR THE PREVENTION OR ELIMINATION OF MYCOTOXINS

It is known that different strains of mycotoxigenic fungi differ in their ability to produce mycotoxins. Also, environmental conditions (temperature, gas compositions, substrate constituents, etc.) may affect the production of certain toxins by the various toxin producers. Mycotoxin production is also affected by the presence of other competitive microorganisms that share the same ecological niche with the toxin producer. However, it should be borne in mind that competition might not only result in suppression; it may also have a promoting effect.

Several different approaches have been suggested to cope with the mycotoxin problem in the produce. The most efficient way is to prevent fungal growth, either in the preharvest phase or during the postharvest (storage) phases, by chemical, physical or biological means (Barkai-Golan, 2001), or by an integrated approach in which several different treatments – usually each of them at reduced level – are combined, with the possibility of achieving a synergistic effect. However, since growth inhibition is not always possible, especially during the preharvest period, several strategies for managing mycotoxins that have already been generated in the produce or in the processed food have been investigated. These mainly comprise: removal of contaminated material by extraction of the toxin from the substrate, detoxification by physical means (e.g., irradiation, or heat treatment); adsorption by abiotic carriers such as clays or carbon; binding of the toxins by biotic factors

('biological detoxification'), including live or dead microorganisms or cell suspensions; enzymatic degradation and chemical treatments (Sinha, 1998; Shapira and Paster, 2004). Whichever treatment is used should, however, meet the criteria specified above.

## 18.3. PATULIN IN AGRICULTURAL PRODUCE AND FOOD PRODUCTS

### 18.3.1. INTRODUCTION

Patulin is a mycotoxin produced by numerous species belonging to the genera *Penicillium*, *Byssoschlamys* and *Aspergillus*, but mainly by *P. expansum*, a common pathogen of apples and pears (Frisvad and Thrane, 1996; Delage et al., 2003; Drusch and Aumann, 2005). The toxin may be present, not only in a fresh produce, but also in processed products such as cider or apple juice, as well as other fruit juices (Scott et al., 1972; Stott and Bullerman, 1975; Brackett and Marth, 1979a; Larsen et al., 1998; Beretta et al., 2000; Leggott and Shephard, 2001; Yurdun et al., 2001; Lai et al., 2002; Demirci et al., 2003; Logrieco et al., 2003; Ritieni, 2003; Logrieco et al., 2003; Ritieni, 2003; Tangni et al., 2003; Moake et al., 2005; Katerere et al., 2007). Recently, Karaca and Nas (2006) reported on the presence of patulin in dried figs also. Patulin production increases during the late phase of mycelial growth and the toxin may be degraded over time (Drusch and Ragab, 2003). Toxicological studies of patulin have revealed that the toxin induces, among others, gonotoxic, cytotoxic and immunosuppressive effects (Speijers, 2004).

### 18.3.2. STRATEGIES TO COPE WITH THE PROBLEM OF PATULIN

#### 18.3.2.1. Fruit storage and sorting of damaged produce

Although *P. expansum* can grow and produce patulin at 0°C, Morales et al. (2007a) reported that the toxin was not produced in artificially inoculated apples during storage for 6 weeks at 1°C, although lesions were evident. Patulin was accumulated when the fruits were kept for a further 3 days at 20°C. Patulin accumulation tended to be higher when initial lesions were bigger and no significant amounts of patulin were found in apples with lesions up to 2 cm after cold storage (Morales et al., 2007b). Therefore, keeping the exposure time of apples to warm temperatures as short as possible will reduce the risk of patulin presence in the final produce.

Using damaged produce that might contain patulin could result in the presence of the toxin in the processed foods or drinks. Kadakal and Nas (2002a) produced juice from apples that they had classified according to the proportion of the fruit surface showing decay, and found high patulin contents in juices prepared from 60 to 100 percent decayed apples, compared with

those in juice prepared from sound or from 30 percent decayed fruits. Sorting of damaged fruits, suspected to contain patulin, and careful culling before processing have been recommended as a means to prevent the presence of patulin (Lovett et al., 1975a; Jackson et al., 2003).

Several studies have shown that patulin, produced in the rotten areas, may migrate to other tissues of the fruit, so that 'healthy' – not rotten – tissues may also contain the toxin (Beretta et al., 2000; Laidou et al., 2001; Martins et al., 2002). However, there was no correlation between the concentrations of patulin and citrinin and the size of the rotten area (Martins et al., 2002), and in some cases very high contents of patulin were detected in apples with small affected areas (Beretta et al., 2000). Other studies (Taniwaki et al., 1992; Rychlik and Schieberle, 2001) found that the diffusion of the toxin was restricted to within 1–2 cm of the infected zone. Hasan (2000) has divided infected apple fruits into two portions: the rotten lesions and the non-rotten part, attached to the lesions, and found that patulin content of the lesions ranged from 500 to 900 µg/g, whereas that of the other part ranged from 150 to 400 µg/g. Rychlik and Schieberle (2001) concluded that patulin could easily penetrate through low-viscosity foods containing large amounts of water, for example, blue berries and grapes, whereas in firm and highly viscous foods, such as apples, patulin diffusion was restricted. Therefore cutting off tissue at a distance of 2 cm from the moldy zone is sufficient to remove hazardous amounts of patulin. In summing up the accumulated evidence concerning the ability to avoid patulin in the final product, it can be postulated that foods prepared from low-quality fruits may contain high levels of patulin, and also of citrinin, but trimming away rotten portions of the fruits can reduce, although not totally eliminate, high levels of mycotoxins in the product (Sydenham et al., 1995, 1997; Leggott et al., 2000; Marín et al., 2006a) even if the rotten area is small.

#### 18.3.2.2. Biological elimination

Numerous studies have addressed the use of bacteria, yeasts and filamentous fungi in the biological control of fresh-produce pathogens (Droby et al., 2001; Janisiewicz and Korsten, 2002; Schnürer and Magnusson, 2005), including patulin producers (Gourama, 1997; Florianowicz, 2001), but fewer have dealt with the biological degradation of mycotoxins commonly found in the produce. With regard to patulin elimination, Castoria et al. (2002) studied the ability of three yeast strains to metabolize patulin, and found that *Rhodotorula glutinis* exhibited the highest rates of patulin destruction, in both in vitro and in vivo studies, the latter in apple wounds infected with *Penicillium expansum* in the presence of the yeast. The authors concluded that yeast cells present in rotted apples could metabolize patulin or reduce its production. Patulin was degraded during fermentation processes by the yeasts used (mainly *Saccharomyces* spp.). Although the mechanism of degradation is not completely understood, it was clear that the patulin was not adsorbed to the yeast cells, since patulin products remained in the clarified fermented medium.

As noted above, any process causing degradation of a selected mycotoxin should be followed by toxicological studies showing that the products are not toxic and it is crucial to include that information whenever degradation occurs.

### 18.3.2.3. Physical treatments

**a. Modified atmospheres (MA):** Apples and pears are usually stored at refrigeration temperatures and/or under modified atmospheres (in the case of apples) containing 3 percent CO<sub>2</sub>/2 percent O<sub>2</sub>, which serves mainly to delay senescence but also to suppress postharvest decay (Sommer, 1985). Under these MA conditions, patulin production (on certain apple varieties) by several fungal strains can be suppressed or even blocked, although the fungi remain capable of growing (Paster et al., 1995). The use of MA as a means of fruit preservation is directed primarily towards the prevention of fungal growth and the accompanying mycotoxin production, of which the former is not addressed in this chapter. However, since MA is used for storage of fruits, several key points should be mentioned that relate to its use and suppression of patulin production during the transportation and storage of apples and, potentially, other fruits (Yackel et al., 1971; Sommer et al., 1974; Lovett et al., 1975b; Sommer et al., 1981; Sommer, 1985; Sitton and Patterson, 1992; Moodley et al., 2002). (a) Different strains of *P. expansum* may differ in their responses, as expressed in growth and/or patulin production, to the same MA; (b) there is an interrelationship between temperature and MA in their effects on growth and toxin production; (c) the 'substrate' (e.g., fruit cultivar) may also affect both growth and toxin production under certain MA/temperature conditions; and (d) packaging materials, if used, may differ in their effects on fungal growth and toxin production under a given MA.

**b. Gamma irradiation:** Use of gamma irradiation (GI) as a means to suppress the growth of mycotoxigenic fungi or the production of mycotoxins during disease development has been thoroughly investigated (Sharma, 1998; Barkai-Golan, 2001). With regard to patulin production, Bullerman and Hartung (1975) showed that irradiated spores of *P. expansum* produced less patulin than the controls. Adams et al. (1976) reported that at doses of 50 and 100 krad, the amount of patulin produced by *P. expansum* was not affected although growth was partially inhibited; growth of *P. patulum* was also decreased at these levels. However, the fungi produced less patulin after receiving doses of 100 krad. At 200 krad growth and patulin production were decreased for both fungi. The effects of GI on several mycotoxins other than patulin were also studied, and it was found that the dosages needed to destroy selected mycotoxins could cause severe damage to the produce, and these doses were far beyond those permitted to be used in food (Sharma, 1998). The effect of irradiation on the patulin content of apple juice was studied by Zegota et al. (1988), who found that patulin content was reduced by one-half of its initial value by a radiation dose of 0.35 kGy (D<sub>50</sub>), and that its level was not changed following a subsequent 8 weeks of storage at 4°C.

However, during the storage of irradiated samples, a slight increase in the titratable acidity and decreases in the carbonyl and ascorbic acid contents were recorded. The undesirable effects of irradiation on fruit juices and the potential of several means of mitigating them, e.g., by irradiating at low temperatures or adding antioxidants are described by Fan et al. (2004).

#### 18.3.2.4. Patulin elimination during processing

The effect of processing on mycotoxin content in fruit juice has further been elaborated in the present book by Shephard and Vismer.

Patulin may be present in produce destined for processing, and patulin-producing molds could contaminate the juice prior to thermal processing. Since several fungal species or structures, e.g., ascospores, are resistant to heat (Roland and Beuchat, 1984), surviving cells could eventually produce patulin in the final product (Harrison, 1989), and even in refrigerated products, e.g., ciders at 4°C (McCallum et al., 2002).

Processing of fruits to yield various products involves several technological steps, each of which may affect patulin that is already present. Studying the effects of various treatments on patulin and fumaric acid, during the production of apple juice concentrate, Gökmen and Acar (1998) found that both patulin and fumaric acid contents of apple juice decreased during the various steps of the process: washing and handling, milling, pressing, aroma recovery, depectinization, clarification and rotary vacuum. The average removal of patulin during these steps was 61 percent, with the most significant decreases being observed in the washing and handling, and the clarification steps. However, heat treatment applied during evaporation resulted in an appreciable increase in fumaric acid. Patulin content in the samples remained almost the same during evaporation, probably because of the stability of the toxin under heating, at the pH levels found in apples juice (discussed below). Acar et al. (1998) showed that washing and handling appeared to be the most critical steps in removing patulin from apples: up to 54 percent of the mycotoxin could be removed when high-pressure water spraying removed rotten parts of the fruits.

The stability of patulin has been studied under various conditions associated with processing; these include inter alia, pH variations, heat treatments, pasteurization and the presence of food additives. Patulin is unstable at alkaline pH values; Brackett and Marth (1979c) calculated that at pH 8.0, the average half-time for disappearance of the toxin was 63 h, compared with 1308 h at pH 6.0, both at 25°C; they also indicated that the half-life depended on the trial conditions, e.g., the buffer system. The toxin is more stable in acidic solutions and it was found to be resistant to heating up to 125°C at pH ranging from 3.5 to 5.5 (Lovett and Peeler, 1973); destruction times at all the temperatures tested were longer at pH 3.5 than at 5.5. With regard to patulin formation under various pH values, large amounts of patulin have been found to be produced in apple juice at pH 3.2–3.8 (Damoglou and Campbell, 1986). Thus, the food pH can affect both production and destruction of patulin.



Patulin is relatively stable at high temperatures, and its rate of destruction differs between products and depends not only on the pH but also on the product composition, the length of storage and the temperature applied. With regard to the product composition, sulfhydryl (SH) compounds such as cysteine, thioglycolate and thiosulfate are known to inactivate patulin, and covalent interactions with amino groups in enzymes were also demonstrated (Scott, 1994).

Ciegler et al. (1975) attributed the disappearance of patulin from foods to the reaction of the toxin with sulfhydryl-containing amino acids and proteins to form uncharacterized adducts; they demonstrated that patulin–cysteine adducts retained teratogenic activity in chick embryo tests.

Scott and Somers (1968) indicated that patulin stability was correlated with the levels of SH in the product: it was more stable in products with low SH content (apple juice) than in those with high SH content (black currant and orange juices). Data presented by Lovett and Peeler (1973) further indicated that thermal processing of low-acid foods, especially those with low sulfhydryl content, may not destroy patulin. With regard to high-temperature treatments, patulin was not destroyed by exposure to 80°C for 10–20 min (Scott and Somers, 1968) or for 30 min (Kubacki, 1986). While testing the effect of thermal treatments on patulin stability, Wheeler et al. (1987) studied the stability of patulin in apple cider by using eight high-temperature/short-time (HTST) treatments (60, 70, 80 and 90°C, for 10 s, 90°C for 20, 40, 80 and 160 s and one batch treatment of 90°C for 10 min). The 60, 80 and 90°C HTST treatments and the 90°C batch pasteurization (10 min) treatment resulted in a significant reduction in patulin concentration. However, the thermal processing was not sufficient to ensure a toxin-free product. Kadakal and Nas (2003) reported that the concentration of patulin decreased only partially in apple juice heated (90 or 100°C) under atmospheric pressure, or subjected to evaporation (at 70 or 80°C) for 20 min. While studying the thermal stability of patulin, Kubacki (1986) found that the toxin was stable at up to 80°C for 30 min. It slowly started to decompose slowly at 90°C, and over 20 percent of the compound disappeared in 30 min. at 120°C. Thus, patulin can occur in juices that have undergone pasteurization processes (Harrison, 1989; Anonymous, 1990; McKinley and Carlton, 1991).

At moderate temperatures of 20–30°C, the patulin level in apple juice stored for 10 days at room temperature did not decrease (Pohland and Allen, 1970). In apple juice stored for up to 3 weeks at 22°C there was only a slight decrease in patulin content, but a high reduction was noticed in canned apple juice after 5 weeks of storage (Scott and Somers, 1968). Storage of pasteurized cider for 1 month at 20–22°C had no effect on the patulin content (Wheeler et al., 1987). Koca and Ekşi (2005) studied the effects of two storage temperatures (22 and 30°C) on the patulin content in apple juice concentrates during 6 months of storage. A reduction to below detectable limits was found after 4 months of storage at either temperature. The sharpest decrease in patulin levels was observed at the beginning: reductions after 1 month of storage at 22 and 30°C were 45–64 and 66–86 respectively, and after one additional

month the reductions were 75–86 percent and 78–100 percent respectively. This indicates that the degree of patulin disappearance is dependent not only on the storage conditions, but possibly also on the initial toxin concentration in the substrate tested.

Various methods and several different yeast strains are used to ferment apple juice, and the stability of patulin during the process was studied. Harwig et al. (1973) reported that the concentration of patulin in non-inoculated apple juice declined by about 50 percent after it had stood for 2 weeks. Disappearance of patulin from fermenting juice was more rapid than from non-inoculated juice, and little or no patulin was detected after 2 weeks of fermentation. However, the rate of disappearance of patulin during the fermentation was dependent on the yeast strain used.

Stinson et al. (1978) used eight yeast strains in evaluating the stability of patulin during typical American processes of fermentation, and found that patulin added to apple juice was completely destroyed by every strain during fermentation of the juice to apple wine, and by six of them during fermentation to hard cider. Significant reduction of patulin following fermentation was reported also by Burroughs (1977), who used two strains of *C. cerevisiae* that are commercially applied in English cider making. There was no indication that the rate of disappearance of patulin was related to the rate of yeast growth, or that the disappearance might be due to a possible reaction between patulin and the metabolites secreted by the yeasts. Although the mechanism of patulin reduction is not clear, the authors suggested that the rate of disappearance may depend to some extent on the flocculation pattern of the strain used. Another factor which was considered to affect the rate and extent of patulin destruction is the inhibitory effect of patulin on the yeast cells. Burroughs (1977) found that although most of the growth of the yeasts used (*C. cerevisiae*) occurred in the first 24 h of fermentation, the patulin had almost disappeared after 3 days, thus indicating that the decrease in patulin occurred as a result of fermentation and was not dependent on the yeast growth. Brackett and Marth (1979b) also reported that there was no appreciable effect of the presence of patulin on alcohol production, at concentrations up to 500 µg/l, and that the toxin did not inhibit yeast growth. However, when adding various amounts of patulin, up to 200 mg/l, to the fermenting medium, Moss and Long (2002) found that there was a concentration-dependent inhibition of yeast growth, although, following an increased lag phase, the yeast could grow even in the presence of the highest concentrations of patulin used, which were higher than those normally found in naturally contaminated apple juices. The inhibitory effect depended on the medium used. It seems that although patulin may affect yeast growth to some extent, which depends on the patulin concentration and the medium tested, the toxin is almost completely destroyed during fermentation.

Various studies aimed to characterize the products resulting from patulin degradation during fermentation. Stinson et al. (1979) tried to confirm that the formation of adducts with compounds containing sulfhydryl groups could account for the disappearance of patulin, and found that much of the patulin – about 60 percent – was converted to water-soluble substances, which were not

identified under the trial conditions, other than adducts of cysteine, peptides or proteins containing sulhydryl groups. However, the chemical compositions and the toxicological properties of the products were not identified. Further studies have identified that the major metabolite resulting from patulin degradation was (E)-ascladiol, which can be further degraded to (Z)-ascladiol (Sekiguchi et al., 1983; Moss and Long, 2002).

Differences in fruit quality, and between methods of fermentation, initial patulin contents in the raw material and the strains of yeast may account for the differing findings concerning the stability of patulin during fermentation. However, it can be stated that most of the studies found that large amounts of patulin were destroyed during fermentation and, therefore, the toxin is not found in alcoholic fruit beverages or vinegars produced from fruit juices. Patulin removal may be achieved during fruit processing, by extraction with solvents or water, or by adsorption (binding) onto materials used in the processing chain or that are added specifically for this purpose. Sydenham et al. (1995) showed that the patulin level in non-processed apples dropped significantly following an initial water treatment, and analysis of the wash water showed that appreciable levels of the toxin had been transferred from the solid to the aqueous phase. The efficacy of high-pressure water spraying in removing patulin from apples, by removal of rotten parts, was reported also by Acar et al. (1998). Binding of patulin to an adsorbant, usually during processing, seems to be an appropriate strategy to cope with the problem of patulin in ciders, juices, etc., therefore, several studies aimed to measure the ability of activated charcoal to remove patulin. Sands et al. (1976) found that patulin was entirely removed from cider by shaking with activated charcoal, added at 20 mg/ml, or by elution through a 40- to 60-mesh charcoal column. The authors were able to reduce the patulin in naturally contaminated cider below detectable levels by using charcoal at 5 mg/ml. Although agitation with charcoal and filtration through the material were highly efficient in removing patulin, drastic reductions in cider color were observed. Thus, although patulin is adsorbed by charcoal, other types of this material, as well as other adsorbants, which do not affect cider quality, should be found. Leggott et al. (2000) reported that a combined depectinization/charcoal/ultra-filtration stage significantly decreased patulin concentration, probably by adsorption of the toxin on the activated charcoal, as was shown also by Kadakal et al. (2002) and Kadakal and Nas (2002b). Steam-activated carbon could also adsorb patulin from juices, but large amounts of carbon were needed in juices at high Brix levels (Leggott et al., 2001). Huebner et al. (2000) prepared composite carbon adsorbent (CCA) by bonding ultrafine activated carbon onto granular quartz to produce a carbonaceous adsorbent with both a high surface-to-volume ratio and good adsorbent bed porosity and handling characteristics. When used in fixed-bed adsorption columns, CCA exhibited much higher capacities for adsorption of patulin from aqueous solution than from apple juice, probably because of the presence in the juice of other solutes that compete for adsorption sites. However, patulin adsorption capacities were increased

when larger bed volumes of CCA were used. The breakthrough capacities (50 percent patulin elution) on 1.0-, 0.5- and 0.25-g CCA columns were approximately 14, 4 and 2 ml (equivalent to 137.5, 38.5 and 19.9 ppb of patulin, respectively). The adsorption kinetics of patulin on activated carbon were determined from equilibrium adsorption isotherms and adsorption rates over the temperature range of 20–80°C (Mutlu et al., 1997). It was found that the adsorption of patulin on the activated carbon surface occurred by means of physical mechanisms only, and that the apparent adsorption rate of the toxin increased as the temperature and the initial patulin concentration increased. The data presented could be useful to industrial enterprises that are considering the addition of activated carbon for patulin removal.

Treatment of apple juice by fixed-bed CCA adsorption at various temperatures revealed that patulin adsorption increased with temperature. A good correlation that was found between reduction of patulin levels from aqueous solution, on the one hand, and toxic effects in *Hydra attenuata*, on the other hand, served as a bioassay to confirm the effectiveness of CCA for removal of patulin from the solution. Although the findings indicate that CCA used in a continuous-flow, fixed-bed application effectively reduced patulin levels in both aqueous solutions and naturally contaminated apple juice, the authors reported that the appearance and taste of the juice may be affected by treatment with activated carbon adsorbents (Huebner et al., 2000).

Clarification is used to remove solid bodies from the liquid medium. It can be achieved, for example, by using pectinase enzymes, which break the pectin coat of the protein particles and so cause their sedimentation; by addition of materials such as bentonite and gelatin; by centrifugation; or by filtration.

Apple juice clarification techniques that used gelatin, bentonite and activated charcoal decreased patulin levels but had adverse effects on the juice, whereas adsorbent resin treatments that followed ultrafiltration reduced patulin levels without affecting juice quality (Gökmen et al., 2001). Clarification using a rotary vacuum precoat filter was more effective in removal of patulin from the juice as compared with ultrafiltration alone (Acar et al., 1998). Bissessur et al. (2001) used four clarification processes – fining with bentonite, enzyme (pectinase) treatment, paper filtration and centrifugation – and four combination treatments to evaluate the removal of patulin during the clarification processes involved in apple juice manufacture. Centrifugation was the most effective clarification procedure, with patulin removal of 89 percent. However, this value included patulin that was removed in the pressing step, as well as the amounts lost in the extraction and the clean-up steps. The patulin reduction by the centrifugation alone was calculated to be 20.5 percent. Fining was the second most effective clarification process, and filtration and enzyme treatment were less effective than the other two treatments. Since patulin was recovered from the centrifugation pellet and the sediment from the fining process, Bissessur et al. (2001) suggested that the removal of patulin could be attributed to adherence of the toxin to solid particles. The combination treatment did not achieve greater percentages of

patulin removal than either means alone: centrifugation and fining, which formed the most effective combination for patulin removal, achieved the same rate as centrifugation alone.

The interaction between patulin and the solid parts of cloudy apple juices was described also by Baert et al. (2007) and was explained by the interaction of patulin with the proteins present in the solid parts of the juice. The authors suggested that the interaction is probably based on the nucleophilic attack on the double-bond conjugated with the carbonyl function according to a 1,6-addition, and concluded that it seems unlikely that this covalent binding could be broken during extraction. In light of the data presented above, it can be concluded that a significant amount of patulin can be removed during the processing steps. At each step, small amounts of patulin seem to be removed, which vary according to the process. However, the total amount of patulin removed in the course of the whole chain ensures that the product will be almost free of patulin.

The effects of the vitamins thiamine hydrochloride (TH), pyridoxine hydrochloride (PH) and calcium d-pantothenate (CDP) on patulin in apple juice were studied by Yazici and Velioglu (2002). Patulin was fully degraded in samples stored at 22°C for a period of 6 months. However, the quality parameters diminished significantly. Application of CDP at 1000 and 2500 mg/kg resulted in patulin reductions of about 74 and 94 percent, respectively, without any considerable reduction of other quality parameters. Addition to the AJC of TH at 1000 mg/kg, PH at 625 or 875 mg/kg, or CDP at doses of 1000 and 2500 mg/kg followed by storage at 4°C for 6 months resulted in reductions of about 55–68 percent, compared with about 36 percent for the control. No reductions in quality parameters were recorded. In food products containing different additives, patulin elimination depends, among other factors, on the temperature and duration of storage. Studies that aim to elucidate the efficacy of a method or a material in patulin reduction should also address the effect on the quality of the test medium.

Patulin reacts with SO<sub>2</sub> and could, therefore, be destroyed by the free SO<sub>2</sub> present in cider or when SO<sub>2</sub> is used as an additive, i.e., as an antioxidant or preservative agent. Pohland and Allen (1970) reported that patulin in apple juice was not stable in the presence of SO<sub>2</sub>, and reacted rapidly with quite low concentrations of the material. Burroughs (1977) found that in 10<sup>-3</sup> M bisulfite (64 ppm SO<sub>2</sub>) containing a ten fold excess of SO<sub>2</sub> per mole of patulin, which itself was at concentrations higher than those found in apple juice, there was no detectable decrease in the patulin peak. Further experiments revealed that the affinity of patulin to SO<sub>2</sub> was pH-dependent and was of little significance at the SO<sub>2</sub> concentrations used in the processing of apple juice and cider, i.e., below 200 ppb. The author concluded that SO<sub>2</sub> cannot be regarded as an effective means to reduce patulin in products.

The effect of ascorbic acid or ascorbate, which are commonly added to beverages, on patulin was studied mainly with regard to apple juice. Brackett and Marth (1979d) found that, in the presence of ascorbic acid, the amounts of patulin in buffer solution and in apple juice decreased, but the toxin was more

stable in the juice. The authors suggested that there was a metal-catalyzed oxidation of ascorbic acid or ascorbate, which was also tested, that yielded a single oxygen or free-radical forms of a metal-ascorbate complex. The single oxygen generated by a series of reactions could attack the conjugated double bond of patulin. The addition of ascorbate or ascorbic acid to apple juice that contains low levels of patulin or to juices that are stored for several weeks before consumption seemed to be an effective strategy to remove patulin. However, Brackett and Marth (1979d) stated that before that strategy is applied, work should be done to prove that the patulin residues resulting from the reaction are not toxic, and to exclude the possibility that the reaction is reversible so that patulin could be 'regenerated'.

Drusch et al. (2007) reported that at acidic pH, the presence of ascorbic acid reduced the stability of patulin; they supported the mechanism suggested by Brackett and Marth (1979c), and noted that patulin is decomposed by free radicals generated by the oxidation of ascorbic acid to dehydroascorbic acid.

Both free radicals, such as hydroxyl radicals or single oxygen, and free radical metal ascorbate complex are involved in the degradation of patulin. Since patulin degradation does not occur after complete oxidation of the acid, the addition of ascorbic acid to apple juice, prior to filling, as a means for decontamination was not recommended by Drusch et al. (2007) since the oxygen content in the headspace of the package is limited. A patulin elimination treatment that combines ascorbic acid with an absorbent polymer was suggested by Canas and Aranda (1996), who found that chemical degradation plus physical removal decreased the patulin content in apple juice by about 85 percent. Thus, integrating methods seems to offer a promising and feasible means for coping with the patulin problem in the food chain.

## 18.4. OCHRATOXIN A IN GRAPES AND IN WINES

### 18.4.1. INTRODUCTION

Ochratoxins (A, B, C and D) are a group of chemically related toxins that consist of an isocoumarin linked to L- $\beta$ -phenylalanine. The most predominant toxin of the group is ochratoxin A (OTA), which has been found worldwide, in coffee, cereals, grapes, and their products. Among its toxic activities, OTA exhibits nephrotoxic, genotoxic and immunotoxic properties (Benford et al., 2001). Many fungal species isolated from grapes, e.g., *A. carbonarius*, *A. fumigatus*, *A. niger*, *A. ochraceus*, *P. verrucosum*, and *P. pinophilum*, are capable of producing OTA in grapes. However, among the known producers of OTA in grapes, *A. carbonarius* has been proved to be the main one, therefore, this fungus is considered as the main source of OTA in wines and vine fruits, especially in regions with hot and dry climates (Pitt, 2000; Battilani and Pietri, 2002; Cabañes et al., 2002; Da Rocha Rosa et al., 2002; Abarca et al., 2003; Battilani et al.,

2003a; Serra et al., 2003; Magnoli et al., 2004; Sage et al., 2004; Serra et al., 2005; Bejaoui et al., 2006a; Bellí et al., 2006a; Gómez et al., 2006; Guzev et al., 2006; Serra et al., 2006a; Tjamos et al., 2006; Leong et al., 2007;). The high rate of appearance of *A. carbonarius* in hot and dry regions is attributed to the resistance of the black *Aspergilli* to sunlight and ultraviolet light (Benford et al., 2001) whereas in colder countries, *Penicillium* species are considered to be the source of OTA contamination.

## 18.4.2. CONDITIONS FOR GROWTH IN VITRO

OTA-producing fungi can invade the grapes at the preharvest stages. The main factors affecting fungal growth and mycotoxin production in vivo include environmental conditions, e.g., water availability, temperature and photoperiod; the composition and condition of the substrate, i.e., in the case of OTA in grapes, stage of maturation; competition among the microorganisms present on the substrate; and fungal factors, e.g., ability to colonize the substrate and to produce the mycotoxin. In order to elucidate the environmental conditions that affect OTA production, in vitro studies were carried out, mostly with *A. carbonarius*, *A. ochraceus* and *Penicillium verrucosum*. Using an artificial grape juice medium, Magan and Aldred (2005) found that the optimum growth of *A. carbonarius* occurred at 30–35°C and water activity ( $a_w$ ) of 0.98–0.99, whereas the most favorable ranges for OTA production were 15–25°C and 0.93–0.95, respectively. Working with 10 *Aspergillus* isolates of the *Nigri* section, obtained from grapes, Bellí et al. (2004a) reported that maximal growth rates occurred between 30 and 37°C, with 30°C being the optimal for the growth of *A. carbonarius*. For all the isolates and temperatures assayed, growth rates increased with increasing  $a_w$ , with the optimum at 0.98 $a_w$ . In another study, Bellí et al. (2005a), showed that OTA production by *A. carbonarius* was also favored by high  $a_w$ . However, although OTA can be produced at the optimal growth temperature, the maximum amount of the toxin was recorded at 20°C, which is below the optimal for growth. Differences between isolates in their optimal temperature and  $a_w$  conditions for growth and for OTA production were reported also by Leong et al. (2006a), who studied the effect of  $a_w$  and temperature on growth and OTA production by five strains of *A. carbonarius* and two strains of *A. niger* isolated from Australian vineyards. Since temperatures of around 37°C may prevail in the field at harvest time, when the  $a_w$  of grapes in the field is 0.98, Bellí et al. (2005a) and Leong et al. (2006a) concluded that field conditions may favor the growth of the OTA-producing fungi from veraison until harvest. The rate of OTA production and its level depend not only on environmental conditions but also on the incubation period, as was reported by Marin et al. (2006b), who studied the effect of incubation time, up to 10 days, and temperature, between 7 and 42°C, on *A. carbonarius* growth and OTA production. Growth and OTA production occurred only within the range of 15–35°C, and OTA accumulation peaked after 10 days of incubation at all temperatures except



30°C, at which the maximum was detected after 6–8 days. In general, increasing the incubation temperatures resulted in earlier production of the toxin. However, at the optimal growth temperature (35°C), OTA was rarely detected, which again indicated that rapid or maximal growth does not correspond to maximal OTA production, regardless of the incubation time (Leong et al., 2006a).

It should be noted that possible inconsistencies among findings about optimal growth conditions and OTA production may result from differences between strains (Bellí et al., 2004a; Mitchell et al., 2004).

In order to predict the influence of field conditions on the growth of ochratoxigenic fungi and on OTA production, Bellí et al. (2006b) studied the effects of a 12-h photoperiod and 12-h alternations of temperature, simulating day (28°C) and night (20°C) field conditions, on the growth of three ochratoxigenic strains of *A. carbonarius*. They found that the photoperiod, rather than the temperature variation, may stimulate the growth. Fluctuating temperature and photoperiod did not have any significant effect on toxin production by the isolate. Since the changes in the temperatures and the photoperiod that occur in the field may differ from those used in the study, and, in addition, other parameters such as humidity and agricultural practices could affect growth and OTA production, Bellí et al. (2006b) concluded that predicting OTA formation in the field was 'far from possible'. Working with isolated berries that were artificially inoculated with ochratoxigenic *A. ochraceus*, Pardo et al. (2005) studied the influence of relative humidity (RH) and temperature on fungal growth and OTA production. Visible growth was maximal at 30°C and 80 and 90 percent RH. However, strains differed in their ability to produce OTA under the RH/temperature conditions tested, and the ranges of these parameters in which OTA production occurred were narrower than those for fungal growth. Based on the results, predictive response surface models (RSMs) to determine the percentage of berries with visible *A. ochraceus* growth under unfavorable RH were developed. Other predictive models for fungal growth, which might be extended also to predict the rate of OTA accumulation, have also been presented. These models can assist in identification of the critical control points during growing and processing of the produce (Pardo et al., 2006).

### 18.4.3. OCHRATOXIN A IN GRAPES AND WINES

The presence of OTA in wine has been reported worldwide during the last decade (Bellí et al., 2002; Jørgensen, 2005; Blesa et al., 2006; Varga and Kozakiewicz, 2006). The toxin can appear in several kinds of wines made from grapes grown in various geographical zones under diverse climatic conditions and agricultural practices, as illustrated by several examples given below.

The presence of OTA in Italian, but not in Hungarian, wines was reported by Brera et al. (2005). While analyzing 116 samples of wine from Valencia (Spain), Blesa et al. (2004) found OTA at levels ranging from <0.01 to 0.76 ng/ml. Mankotia et al. (2004) determined OTA levels in 251 samples of wines and



grape juices collected over 3 years in Canada: the OTA percentages were about 30 percent in red and white wine samples, and 6.5 and 4 percent in red and white grape juices, respectively. Wines from the United States contained no quantifiable levels (20 and 40 pg/ml in white and red wines, respectively) of OTA. A study by Ana et al. (2005) found that none of the analyzed wines produced in Argentina and Chile were contaminated with OTA; however, the authors indicated that exposure to OTA that might be present in wine in these countries should not be ruled out, and they recommend that more studies should be performed. Chultze et al. (2006) found very low levels of OTA in wines of Brazilian, Argentinian and Chilean origin. In Turkey, wines from two regions (Aegean and Thrace) were much more contaminated with OTA than those from other regions (Anli et al., 2005). OTA was reported also in wines produced in Taiwan (Lin et al., 2005) and Morocco (Filali et al., 2001). Red wines – mainly European ones – have been found to contain greater amounts of OTA than white wines (Zimmerli and Dick, 1996; Visconti et al., 1999; Ottener and Majerus, 2000; Bellí et al., 2004b; Lo Curto et al., 2004; Mankotia et al., 2004; Rosa et al., 2004; Anli et al., 2005; Drusch and Aumann, 2005; Battilani et al., 2006a; Blesa et al., 2006). However, there were no apparent differences between OTA levels in red and white wines in Australia (Leong et al., 2006b) or in South Africa (Standar and Steyn, 2002). As for the differences in OTA between red and white wines, the reasons are primarily related to their differing wine-making procedures. For example, during red wine processing, after mashing, the skin and the juice are put aside for several days without fermentation and under aerobic conditions, which allows the growth of molds and the subsequent OTA production (Majerus et al., 2000; Ottener and Majerus, 2000). Also, more clarifying steps are used throughout white wine vinification, when OTA is bound to fining agents (described below). Special wines, such as liqueurs, may contain even more OTA than red wine, possibly because grapes destined for those wines are left on the vine for over-ripening or are exposed to the sun for extended periods after harvest, and both of these practices increase the exposure of the grapes to fungal attack (Gómez et al., 2006). Grape products other than wine, e.g., juice, wine vinegar and must, were also found to contain OTA (Burdaspal and Legarda, 1999; Majerus et al., 2000; Larcher and Nicolini, 2001; Markaki et al., 2001; Bellí et al., 2002; Sage et al., 2002; Bellí et al., 2004c).

In order to better understand the factors involved in OTA production in the field, the populations of OTA producers in the berries at different maturation stages, usually from setting until harvest, and the toxin contents in the developing berries were evaluated in various geographic areas and under diverse climatic conditions.

The fungal population increases as the grapes mature or during the initial stages of drying (Leong et al., 2004; Valero et al., 2005; Gomez et al., 2006; Guzev et al., 2006; Leong et al., 2006b), and the increased fungal development contributes to OTA contamination in the last development stages of the berries (Bau et al., 2005). The period between early veraison and harvesting can, therefore, be regarded as the critical period for OTA accumulation, which

varies according to the meteorological conditions prevailing during that period (Battilani et al., 2006a). However, fungal counts differed between the varieties tested (Battilani et al., 2004; Guzev et al., 2006; Leong et al., 2006c), and different regions may also differ in the dominant toxigenic species on their grapes, because of their differing climatic conditions (Serra et al., 2003, 2006b). It is quite evident that OTA contents in wines from the warmer, southern regions are almost always higher than those in wines from the cooler, northern growing areas (Zimmerli and Dick 1996; Ottener and Majerus 2000). However, in Australia, wines from warmer regions did not show greater OTA incidence than those from cooler parts (Hocking et al., 2003).

Pietri et al. (2001) analyzed a total of 96 red wines and 15 white dessert wines produced in 19 Italian regions in 1995–97, and found that those from southern Italy contained higher levels of OTA than those from central and northern parts. These findings indicate that the geographic region of origin has a strong influence on OTA contamination, with high toxin levels being produced under warm climatic conditions. Battilani et al. (2003b) studied the effects of the environmental conditions of different regions, i.e., southern and northern Italy, on the dynamics of fungi and on OTA content on grape berries during 1999–2000. They found that *aspergilli* and *penicillia* were already present on grapes at setting, and the populations increased towards early veraison and ripening. They found OTA in grape bunches at early veraison, with increasing amounts at ripening, only in vineyards in southern Italy, and only in 1999. They concluded that the temperature, which was higher in the south than in the north, as well as rain and relative humidity, since 1999 was a wetter year, were the main factors affecting OTA production. Differences in OTA between years, even in the same vineyards managed in the same way, support the hypothesis that meteorological differences between years and between growing areas may be the key factors governing OTA production (López de Cerain et al., 2002; Battilani et al., 2003b).

Sage et al. (2004) isolated the fungal microflora from grapes at two development stages – end of veraison and harvest – in several regions in France. Generally, more diversified mycoflora was found at the end of veraison than at harvest, with the diversity depending on grape variety, maturity, cultural practices, and climatic and geographic conditions. *Penicillium* spp. were predominant in the northern vineyards, whereas southern vineyards were generally more contaminated by *Aspergillus* spp. *A. carbonarius*, which can produce OTA, was isolated only in one southern area (Languedoc-Roussillon), and OTA was detected in musts of red wines from that area. The results confirmed that the Languedoc region was a risk area among French vineyards. Another survey conducted in France over 3 years revealed that, regardless of sampling year, 32.5 percent of the fungal isolates isolated between setting and harvest were OTA producers; 93 percent of them were black aspergilli, and *A. carbonarius* was the main OTA producer (Bejaoui et al., 2006a). The results of that survey further confirmed that grapes from Languedoc-Roussillon had the highest OTA concentration among French grapes. The relationship between the incidence of black aspergilli and the thermo-wetness maps has been shown by Battilani et al.

(2006b) in several Mediterranean countries that experienced high incidence of fungi in hot and wet years. Since meteorological conditions play major roles in both fungal colonization and OTA presence, Battilani et al. (2006c) used the summation of degree/day and rain in the period between 21st August and 10th September as the basis of a model that seems to be promising in identifying geographic areas where the risk of OTA presence is high, low or absent.

A positive correlation between the incidence of OTA producers and the temperature in the field was presented by Belli et al. (2005b), who indicated that the most contaminated Spanish wine grapes were those collected from the warmest region. However, no significant correlation between OTA production and other climatic conditions, e.g., relative humidity or rainfall, was evident. In addition, no OTA was found in musts in any sampling year, despite the high percentage of *A. carbonarius* strains capable of producing OTA found in the grapes. In Portuguese wine grapes, most of the OTA-producing strains were isolated at harvest time; OTA was detected in grapes at three maturation stages: pea berry, early veraison and ripe berry. The OTA production by *A. carbonarius* was related to the composition of the berry: it was correlated positively with total acidity and negatively with reducing sugars content. OTA can be produced already at early maturation stages (Serra et al., 2006c).

Differences in fungal invasion to the grapes are related not only to the climatic conditions, the agricultural practices and the stage of maturation of the berries, but also to the condition of the grapes and the aggressiveness of the fungi. The relevant agricultural practices comprise mainly preharvest treatments with pesticides, herbicides and fertilizers, and many reports address the effect of preharvest treatment on OTA content in grapes. However, this subject is outside the scope of this chapter.

In studies which included artificial inoculation, damaged berries inoculated with *A. carbonarius* contained more OTA than those inoculated with *A. fumigatus*: the former colonized and penetrated even those berries without skin damage, whereas the latter colonized only the pulp in damaged berries (Battilani and Pietri, 2002). Leong et al. (2006c) also reported that bunches with no berry damage yielded *A. carbonarius*, although at low levels. *A. carbonarius* and *A. niger* are saprophytes, which penetrate the fruit tissue mainly through existing injuries, that are most likely to occur at harvest time (Benford et al., 2001; Serra et al., 2005). Factors that spoil grapes are: insects, pathogenic fungi, mechanical injury, excessive irrigation and rain. Leong et al. (2004) found that the severity of infection with members of *Aspergillus* section *Nigri* was highest in seasons when grapes barriers were rain damaged. However, *A. carbonarius* was present even in undamaged grapes, and the toxin may occur in grapes that have no visible symptoms (Battilani and Pietri, 2002; Serra et al., 2006a; Leong et al., 2007). It is not clear whether the OTA in those grapes was produced during an early stage of infection, before the appearance of the symptoms, or was translocated from infected grapes (Leong et al., 2007). However, the amounts produced in grapes with no visible fungal growth were much less than those produced in damaged grapes. Thus, OTA may be a particular problem in wine prepared from poor-quality grapes, therefore, efforts should be devoted to minimizing injuries

caused in the field, especially in the period between veraison and harvest, and/or to trimming away the poor-quality, i.e., shriveled, damaged or infested berries. Visual inspection of bunches for *Aspergillus* rot, with special attention to the level of bunch infection, may assist in the identification of vineyards at high risk of OTA contamination (Leong et al., 2007). It should be noted that removing the damaged grapes may contribute only to reducing the risk of OTA contamination; it cannot ensure its total absence from the produce, since, as mentioned above, OTA may be transferred from infested to healthy grapes, and may be present in undamaged berries.

It can be concluded that OTA contamination of grapes and wines can be regarded as a worldwide problem. Differing findings regarding the presence and amount of the toxin in the produce arriving from a given area in different seasons can be attributed to, among other factors, changes in fungal populations, climatic conditions and agricultural management, which may affect fungal growth and toxin production.

#### 18.4.4. BIOLOGICAL DETOXIFICATION

The fate of OTA during processing of various foods has been studied mainly in connection with grain processing. The toxin is relatively heat stable and was not completely eliminated by roasting of green coffee beans, brewing, extrusion or baking (Scott, 1984; Scudamore, 2005). However, the stability of OTA depends upon various factors, e.g., moisture content and pH; it was found stable to gamma radiation also, and was not destroyed even at 7.5 Mrad (Paster et al., 1985). Biodegradation is a promising means to cope with mycotoxins, and it has attracted interest in connection with OTA also.

The microorganism population present in grapes comprises a variety of fungal species, including yeasts (Barkai-Golan, 1980; Zehavi et al., 2000; Sage et al., 2002). Therefore, the interaction between the components of that population is of interest, and studies may result in findings about inhibition of ochratoxigenic fungi and OTA production by means of competition, and also about biodegradation of mycotoxins. Regarding inhibition of OTA production, Valero et al. (2006), studied the interaction between *A. carbonarius* and fungi isolated from grapes and dried vine fruits, which were allowed to grow in pairs. They used Pearson correlation coefficients between OTA accumulation and the *A. carbonarius* colony radius to determine that at  $0.97a_w$  and  $20^\circ\text{C}$ , i.e., the conditions for maximal OTA production, only one fungus (*Trichoderma harzianum*) reduced OTA production. Stimulating or inhibiting effects of the tested fungi on OTA production were recorded at  $0.97a_w/30^\circ\text{C}$  or at  $0.92a_w/30^\circ\text{C}$ , respectively, but these were not the optimal conditions for OTA production. Competing mycoflora can reduce OTA accumulation at  $30^\circ\text{C}$ , and it was suggested that keeping the temperature of grapes, at or above  $30^\circ\text{C}$  during dehydration, may provide some control over OTA accumulation.

Biodetoxification of mycotoxins, by either degradation or binding, which seems to be a promising tool for coping with mycotoxin contamination in foods, and a number of reports on microorganisms capable of degrading mycotoxins have been published (Bata and Lásztity, 1999; Karlovsky, 1999; Shapira and Paster, 2000). Wegst and Lingens (1983) reported on the degradation of OTA to a much less toxic product by a gram-negative soil bacterium (*Phenylobacterium immobile*); they suggested a pathway for the degradation, based on the four metabolites that were isolated from resting cells media supplemented with ochratoxin A. The lactic acid bacteria, *L. plantarum*, *L. brevis* and *L. sanfrancisco*, and the yeast *Saccharomyces cerevisiae* caused an OTA degradation of 40–50 percent, following 24 h of incubation on a flour medium, whereas the mixed population of these microorganisms caused a degradation of 66 percent within the same time. A strong biodegradation activity was observed when a commercial bakery starter was used during sourdough fermentation; OTA degradation reached 97 percent following 24 h of incubation (Piotrowska and Zakowska, 2000).

Shapira and Paster (unpublished data) found that untreated *L. rhamnus* GG (LGG) and *Escherichia coli* K12 (*E. coli*) removed OTA from the growth medium (Phosphate Buffered Saline at pH 7.0), and heat-treated bacteria were much more efficient than acid-treated bacteria or bacterial cell walls. The rate of removal depended on the bacterial concentration, and a maximum OTA removal of about 90 percent was recorded for both of the bacteria at  $10^{10}$  CFU/g. The use of LGG, for OTA removal in the food industry, even after heat treatment, is worth considering, since it removes some other mycotoxins also (El-Nezami et al., 2004; Lahtinen and Haskard, 2004). Biodegradation of OTA by fungi, and especially by those present on grapes, could be of practical importance, therefore, the ability of fungi, and especially the ochratoxigenic black aspergilli, to degrade OTA was studied and efforts were made to identify the resulting compounds. Xiao et al. (1996) reported that, in an *A. ochraceus* culture, OTA was biotransformed into various metabolites such as ochratoxin  $\alpha$  (OT  $\alpha$ ), which lacks the phenylalanine moiety and is less toxic than OTA (Creppy et al., 1983a, b). Out of 70 *Aspergillus* isolates tested for their ability to degrade OTA in yeast extract agar medium, only isolates of *A. fumigatus* and black aspergilli could eliminate the toxin from the medium (Varga et al., 2000). A non-ochratoxigenic *A. niger* isolate that was selected for further studies completely converted OTA to OT  $\alpha$ , during 7 days of incubation, and the latter was gradually degraded to unknown compounds. OTA degradation was faster in solid media than in liquid cultures. Varga et al. (2000) raised the possibility of using *A. niger* or the fungal enzymes involved in OTA degradation for OTA detoxification in vivo. Standar et al. (2000) found one lipase from *A. niger*, out of 23 lipases and esterases screened, that was able to degrade OTA to OT  $\alpha$  and phenylalanine; Pitout (1969) had previously reported the hydrolysis of OTA to the same two substances, by carboxypeptidase A and  $\alpha$ -chymotrypsin.

Using strains isolated from Portuguese grapes, Abrunhosa et al. (2002) reported that most of the strains they tested degraded OTA in vitro, and that the black aspergilli *A. clavatus*, *A. ochraceus*, *A. versicolor* and *A. wentii* were the

most effective, achieving >95 percent degradation of OTA. Strains of *Penicillium* and *Cladosporium* were less efficient: the products of OTA degradation by all tested species of the black aspergilli were the same, although they were not identified. However, different products resulted from OTA degradation by other fungi, which indicated the possible involvement of different enzymes and pathways in the process. It was concluded that OTA can be degraded in the grapes (Abrunhosa et al., 2002). These findings should be taken into account in trying to relate OTA contamination levels to the ochratoxigenic population in the grapes.

Abrunhosa et al. (2006) further studied the ability of several commercial enzymes to hydrolyze OTA and, in light of their findings that fungal strains from the *A. niger* group were highly effective in OTA degradation (Abrunhosa et al., 2002), proteases, mainly from *A. niger*, were included among the commercial enzymes. Of the enzymes tested, Protease A, an acid protease from *A. niger*, and Pancreatine, a mixture of enzymes from porcine pancreas, exhibited the highest degradation capacity: they converted 87.3 and 43.4 percent, respectively, of the OTA to ochratoxin  $\alpha$  after 25 h at pH 7.5 and 37°C. With the same incubation period at the same temperature, but at pH 3.0, only Prolyve PAC, an acid protease from *A. niger*, showed any activity, and that small: it converted 3 percent of the initial OTA to ochratoxin  $\alpha$ . An enzymatic extract from *A. niger* (Ancex), exhibited a higher hydrolytic activity on OTA, and 99.8 percent of the initial toxin was converted to ochratoxin  $\alpha$  after 25 h when incubated at pH 7.5 and 37°C. It exhibited a higher activity at pH 5.6 than the commercial enzymes. However, not all of the oenological enzymes tested were effective in OTA degradation.

Bejaoui et al. (2006b) assessed the ability of 40 *Aspergillus* section *Nigri* isolates to degrade OTA, by using different culturing media. The tested isolates included *A. carbonarius* (a low OTA producer), a non-ochratoxigenic *A. niger*, and *A. japonicus* isolates. All the strains were isolated from French grapes. Removal of OTA was observed for almost all black aspergilli isolates and *A. niger* was the 'best' OTA degrading fungus. However, the degradation capacity of all of the fungi, and even of a given isolate, depended upon the medium used. Furthermore, it was suggested that although OTA was degraded to the less toxic OT $\alpha$ , the latter compound could be further degraded to unknown compounds with unknown toxicological properties. Bejaoui et al. (2006b) suggested that *A. niger*, which has received GRAS (generally regarded as safe) status from the US FDA, can be used for the biological elimination of OTA in grape juices and musts. The possible use of conidia of black *Aspergillus* as adsorbants for OTA in grape juices and musts was suggested by Bejaoui et al. (2005), who used *A. niger*, *A. carbonarius* and *A. japonicus*. All the species used were effective in removing OTA, with *A. carbonarius* being the most efficient. Two stages are involved in OTA detoxification: adsorption of OTA by conidia of the black aspergilli (which could occur also with nonviable, heat-treated conidia) and degradation to OT $\alpha$  (by live conidia only). In further studies the appearance of an unknown compound indicated that the OT $\alpha$  concentration could be decreased by several species belonging to the



*Aspergillus* section *Nigri* (Bejaoui et al., 2006b). In light of these studies, the authors suggested that *A. niger* and *A. japonicus*, which were reported to produce OTA in only a few cases, could be used for OTA removal, since the first of these is considered as GRAS and the second is being used in fungal biotechnology. The use of *A. carbonarius*, the main OTA producer, is more complicated, since OTA-nonproducing strains would need to be selected and carefully monitored during their use. Recently, Peteri et al. (2007) reported that the yeast *Phaffia rhodozyma* was able to convert OTA to OT $\alpha$ , and this conversion is possibly mediated by a cell-bound enzyme related to carboxypeptidases. Chelating agents (like EDTA) inhibited OTA degradation thus indicating that the carboxypeptidase is a metalloprotease, similarly to carboxypeptidase A. *P. rhodozyma* cells, both viable and heat-treated, also were able to adsorb significant amounts of OTA.

Data on means for biodetoxification of OTA seem to be promising, and may lead to the identification of enzymes and genes responsible for OTA detoxification, as was noted by Varga et al. (2005), who found *Rhizopus* isolates highly effective in degrading OTA in vitro and on moistened wheat. These genes could be further transferred into yeasts that are used during vinification, thus utilizing them for OTA destruction during the wine processing. However, the various aspects of biodegradation are not yet commercially applied, but the need to remove OTA from the product is still of the utmost importance. Therefore the fate of OTA during vinification was studied in order to identify the critical points at which the toxin accumulated or persisted. The results of these studies could be useful in the development of food safety programs (Grazioli et al., 2006).

### 18.4.5. EFFECT OF PROCESSING

Damaged berries are highly susceptible to *A. carbonarius* rot, and OTA is concentrated in severely moldy berries (Leong et al., 2007). Wines produced from OTA-contaminated vines had the highest OTA contents (Gambuti et al., 2005). In addition, berries inoculated with *A. carbonarius* are more discolored and shriveled than uninoculated berries and have larger contents of several factors – e.g., total soluble solids of the berries themselves, as well as malic, tartaric and citric acids – that could impair the wine quality (Leong et al., 2006d). Early detection and trimming out of contaminated material could improve the wine quality and contribute to the reduction of OTA levels in wines.

The fate of OTA during the various steps of the wine-making processes was studied especially with respect to the effects of the yeasts used for fermentation and the materials that are applied to improve the wine quality. Arici et al. (2004) used moldy grapes for preparing boiled and concentrated grape juice (Pekmaz) in Turkey and found that the OTA was not destroyed during boiling under low-pH conditions, i.e., about 3.0–4.0, and its content in the final product was five to six times higher than in the initial grape juice because of the concentration component of the processing. Arici et al. (2004) also found that during crushing and maceration, which are the initial stages of the

wine-making process, OTA was released to the product. Gambuti et al. (2005) and Grazioli et al. (2006) also reported that maceration promoted the release of OTA from grape skins, which may serve as OTA carriers. It seems, therefore, that monitoring the OTA content of the crushed and macerated grapes is highly important for assessing its fate throughout the subsequent steps in the process and for predicting the occurrence of OTA in the wine.

The yeasts used in fermentation (*Saccharomyces* strains) are able to reduce OTA levels, and the toxin is reduced during alcoholic and malolactic fermentation (Cecchini et al., 2006; Grazioli et al., 2006). The extent of reduction is inversely related to the initial OTA concentration and is not dependent on the alcohol concentration. In addition, the rate of OTA reduction during fermentation depends on the yeast used for fermentation, the type of wine produced and the yeast concentration (Bejaoui et al., 2004; Cecchini et al., 2006). For example, the rates of OTA removal from white and red wines by several yeasts, as recorded following methanolic extraction of the yeast lees, were about 46–52 percent and 53–70 percent, respectively. Summing up the trend of OTA content from must to wine (at the end of the malo-lactic fermentation), Grazioli et al. (2006) reported that in the first phase, i.e., crushing and maceration, there was an increase of 45 percent in OTA content. In the second phase, i.e., alcoholic fermentation and racking, there was a 30 percent decrease in OTA, and in the third phase, i.e., malo-lactic fermentation and racking, there was a reduction of 57 percent. A decrease of 17 percent in OTA content was observed in bottles of the wine tested after 12 months of storage. In light of these findings, Grazioli et al. (2006) suggested that OTA analysis in the must and at the end of the fermentation would be enough for assessing the OTA content in the wine.

It is worth noting that, since the level of OTA in the finished wine is markedly lower than that found in the grapes, as a result of the toxin removal throughout the vinification, and especially during the fermentation, in which binding to yeast cells occurs (Leong et al., 2006d), monitoring the OTA level is of the utmost importance in unfermented grape products (Zimmerli and Dick, 1996).

With regard to the mechanism of OTA removal during fermentation, the absence of degradation products, the reduction of OTA by acid- or heat-treated yeasts, and the presence of OTA in yeast lees support the hypothesis that the toxin is not degraded but is adsorbed onto the yeast cells (Bejaoui et al., 2004; Moruno et al., 2005; Caridi et al., 2006; Cecchini et al., 2006; Leong et al., 2006e). Ringot et al. (2005) tested the adsorption of OTA onto three yeast industry products: yeast cell wall fraction, purified yeast beta glucan, and a yeast cell wall fraction. The last of these was found to be the best product for OTA adsorption which is based, in this case, on both polar and non-polar non-covalent interactions. In comparing the efficacy of residual yeasts from the alcoholic fermentation of red and white musts, i.e., red and white lees, respectively, to reduce OTA in red wines, Moruno et al. (2005) showed that the OTA reduction obtained from the treatment with the white lees was 20 percent greater than that achieved by using red lees. The difference was attributed to competition on the binding sites between OTA and polyphenols



(elaborated below). Moruno et al. (2005) considered that the technique should not negatively affect the organoleptic characteristic of the wines, but that further sensory evaluation was needed.

Reduction of OTA during vinification is attributed also to its binding to the solid parts of the grapes present (Roset, 2003; Ratola et al., 2005). Fernandes et al. (2003) and Leong et al. (2006d) reported that reduction of OTA occurred at each of the solid–liquid separation stages, with the toxin being discarded with the solid phase (marc and lees). The higher OTA amounts found in red than in white wines were also partially attributed to the role of grape solids in OTA removal, as described by Leong et al. (2006d). In white vinification, grapes are pressed before fermentation and since no alcohol is present, OTA may be poorly partitioned in the liquid phase and may bind more effectively to the grape proteins and solids, which are removed during clarification. In addition, several stages in white vinification that are absent from red vinification, e.g., additional clarification steps, allow higher OTA-binding rates in the white wines. Thus, more OTA is removed during clarification of white juice than during racking of red wines after fermentation (Leong et al., 2006d).

Ratola et al. (2005) reported that OTA concentration was significantly reduced during the microvinification process of Port Wine, the decrease being more pronounced from formation of the initial must to after the start of fermentation. The reduction of OTA is attributed here also to the extensive adsorption of the toxin to the pomace. The efficiency of various clarifying technologies in reducing OTA levels was studied. Microfiltration of red wine through a 0.45- $\mu\text{m}$ , but not through a 0.10- $\mu\text{m}$  membrane, decreased OTA concentration by about 80 percent, indicating that such means are useful for toxin removal (Gambuti et al., 2005). Regarding OTA removal during wine fining, Leong et al. (2006f) noted that the differences between the wine matrix after racking from the gross lees and the juice may affect the efficacy of OTA removal by fining agents. They indicated that OTA and the molecules to which OTA may already be bound are more soluble in wine than in juice, and removal of OTA during wine fining involves more than simple precipitation or filtration of solids. For example, when the efficacy of bentonite in removing OTA from cloudy juice was compared with that of removal from Semillon wine, it was found that the bentonite did not enhance precipitation of OTA from the juice beyond that which occurred due to natural settling. However, a significant decrease in OTA concentration was achieved through addition of bentonite to Semillon wine. Other studies have addressed the efficacy of many enological fining agents in OTA removal, and several mechanisms have been suggested to explain the evident differences between those agents. Castellari et al. (2001) reported that out of 18 agents, including inorganic and organic materials, that they tested, hydrophobic and positively charged fining agents, and especially potassium caseinate and activated carbon, were found to be the most efficient in removing the toxin from red wines. Gambuti et al. (2005) showed that treatment with decolorizing carbon significantly decreased OTA concentrations. However, the use of carbon was associated with a decrease in some important aroma compounds of wine, including 3-methylbutyl acetate, ethyl

hexanoate, ethyl octanoate and geraniol. The first three of these are of primary importance for the aroma of white wines, and geraniol is one of the main volatiles responsible for the aroma of Muscat wines. In addition to bentonite, potassium caseinate reduced the OTA level in Semillon wine, whereas yeast hulls and gelatin were the most effective materials for Shiraz wine. Addition of pectinases had no direct effect on OTA binding to the grape colloids or to other precipitated solids. In every case the extent of the reduction in OTA concentration depended on the amount of the agent added (Leong et al., 2006f).

Addition of SO<sub>2</sub> to juice or of SO<sub>2</sub>-generating agents at the beginning of the vinification did not affect the OTA content (Roset, 2003; Ratola et al., 2005). Several hypotheses have been advanced regarding the mechanisms involved in the binding of OTA to the adsorbent materials, e.g., cell walls and fining materials, and for the observed differences in their abilities to remove OTA. One major factor which limits the efficacy of OTA binding to the added materials is the competition between the toxin and other wine constituents, i.e., polyphenols and proteins, for the binding sites present in the agent; competition that might interfere with the removal of OTA. For example, bentonite is used to bind and precipitate proteins in wine (Castellari et al., 2001), and the proteins would therefore compete with OTA for binding sites on the bentonite particles. Leong et al. (2006f) hypothesized that OTA was bound, by ionic interactions, to the grape-derived proteins that were bound to the bentonite and precipitated from the wine. Proteinaceous fining agents were found to reduce OTA more in Shiraz wine than in Semillon wine, because the larger amounts of grape-derived proteins in the Semillon wine may have competed with the OTA for the binding sites (Leong et al., 2006f). Difference between the quantities of protein in wines may account for the differences in the efficacy of fining agents in removing OTA. However, the competitive interactions between OTA, fining agents, and wine proteins and polyphenols are still not completely clear (Leong et al., 2006f). Adsorption could be attributed to negative charge in the cell walls and the acidic nature of OTA. The cell wall components include polysaccharides, proteins and lipids, which provide sites for adsorption through, inter alia, a hydrogen-bonding hydrophobic interaction. Another possibility for OTA removal during fermentation was suggested by Cecchini et al. (2006), who hypothesized that OTA interacts with phenolic compounds because of amino group ionization of the OTA molecule in the acidic medium, and then further reacts with quinones and other compounds that result from enzymatic and nonenzymatic oxidation of polyphenols during the fermentation. In addition, phenolic compounds interact with metabolic products of yeasts, e.g., mannoproteins. Since the ability of cell wall to adsorb OTA is dependent on yeast macromolecules, including mannoproteins, the interaction between phenolic compounds and mannoproteins yields an increased surface for OTA binding. Castellari et al. (2001) claimed that to achieve adsorption, the interactions between OTA and the adsorbant should be stronger than those between the toxin and the solvent. Binding can be explained in terms of the molecular size and the physicochemical properties of OTA, which is a weak acid (pKa for the carboxyl group of the

phenylalanine moiety is 4.4), and therefore OTA is partially dissociated at the pH of wine, which is about 3.5 and, because of the negative charge that it carries, interactions with materials with positively charged surfaces can occur. Adsorption through the interaction of the phenol moiety and carboxylic group has also been suggested, since the phenol group could be adsorbed onto a negatively charged surface through hydrogen binding and/or charge-transfer complexes. In light of their findings that hydrophobic and positively charged fining materials, e.g., activated carbon and potassium caseinate, were highly efficient in reducing OTA contents in red wines, Castellari et al. (2001) postulated that OTA was mainly adsorbed onto the fining agents by means of the negative charge on the carboxyl group of its phenylalanine moiety. They further indicated that chemically activated carbon is a more efficient adsorbant than steam-activated carbon, since it has a large adsorption surface, because of its irregular surface structure. Several other mechanisms are involved in the interaction between OTA and fining materials, and their elucidation might contribute to improving the process. By measuring the OTA adsorption and the decrease of wine parameters, i.e., contents of total and colored polyphenols, Dumeau and Trione (2000) found that several fining agents that they tested could adsorb not only the toxin but also the wine components, which indicated that polyphenols, which compete for the same adsorption site, were the major compounds in interfering with OTA removal. However, low adsorption of total polyphenols by activated carbon was recorded. It is, therefore, suggested that activated carbon, alone or in combination with other fining agents, might remove OTA from red wine almost completely (Dumeau and Trione 2001).

Fining agents could be classified as useful OTA-reducing materials. However, the efficacy of the materials depends on type of wine being produced. Leong et al. (2006f) stated that two aspects of the solids may be important: the nature of the binding between OTA and the solid, and the amount of solid matter, or of any matter that could be involved. In addition, their use should be thoroughly analyzed to eliminate the possibility of also removing important organoleptic wine constituents.

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